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CONVERTING ENZYME ACTIVITY IN LIVER DAMAGE

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The conversion of the decapeptide angiotensin I (A I) into the octapeptide angiotensin II (A II) is due to the action of a peptidase called the converting enzyme (*Skeggs et al.* 1954), which is believed to be formed in the liver.

According to *Skeggs* (1956) and *Helmer* (1957) the vasopressor activity of A I is weak in relation to that of A II. Therefore the presence and function of the converting enzyme must be considered a prerequisite for the biological activity of the renin-angiotensin system.

The methods available for measuring the activity of the converting enzyme are *Helmer*'s (1957) using isolated strips of rabbit aorta, *Page*'s method (1961) using the isolated rat uterus, and *Wissenbaugh*'s (1964) paper chromatographic separation of A I and A II, as used by *Loyke*.

That hypertension rarely co-exists with an impaired hepatic function has been demonstrated clinically and experimentally by *Raaschou* (1949) in a series of patients with hepatitis and by *L yle* (1960) in a series of alcoholics. *Loyke* (1962) found moreover that patients with renal or essential hypertension became normotensive if they developed cirrhosis of a degree which altered the protein fractions in the blood. In experiments on dogs with renal hypertension *Raaschou & Trautner* (1952) demonstrated that it is possible gradually to lower the blood pressure to a normal level by occlusion of the common duct, and that the decrease sets in a few days after the occlusion. Correspondingly *Loyke* (1964, 1965) found that the blood pressure in rats with experimental renal and desoxycorticosterone induced hypertension could be normalized after a slight liver damage induced by carbon tetrachloride poisoning. This decrease was accompanied by a reduced or abolished conversion activity in the plasma, and *Loyke* suggests that the liver injury is associated with formation of a substance that inhibits circulating converting enzyme. A defective conversion activity was also found in normal rats poisoned by carbon tetrachloride. The liver damage ranged from a mild degree of fatty metamorphosis to pronounced fatty metamorphosis with fibrosis. There were no changes in the other factors of the renin-angiotensin system.

If it is assumed that an increased activity of the renin-angiotensin system is a pathogenetic factor in hypertension, a reduction or abolition of the conversion activity in the plasma can explain the fall of blood pressure observed in the presence of liver damage.

The object of the present study was (1) to work out an assay method for the accurate quantitative testing of the enzymatic activity, and (2) to study this activity both at a normal and at a reduced hepatic function in human subjects and rats.

METHOD

The present method is a modification of *Page*s method which utilizes the fact that A I has a weaker action than A II upon the isolated rat uterus. During the conversion of A I to A II there will be an increase in the action upon the smooth muscles and this will express the degree of conversion.

As a standard incubation the author used

0.4 ml A I (substrate) + 0.1 ml phosphate buffer (2/15 M Na_2HPO_4 + 2/15 M KH_2PO_4 having a pH of 7.4) + 0.4 ml 1.8 per cent NaCl + 0.1 ml plasma containing an unknown amount of converting enzyme.

The mixture was incubated for 15 minutes at 30° C and the enzymatic reaction was arrested by 5 minutes incubation in a boiling water bath. The plasma samples were stored at —20° C in siliconized tubes. The assays were performed on isolated uteri from albino rats weighing about 200 g. The rats were killed by a blow on the head, the two uterine horns were removed and one of these was suspended in a 3.6 ml plastic chamber with one end fixed to the bottom and the other to a horizontal ink writer which traced the contractions with a magnification of 5. The load ranged from 0.8–3 g and the bathing solution in which the uterus was suspended consisted of 0.9 per cent NaCl, 0.042 per cent KCl, 0.006 per cent $CaCl_2$, 0.1 per cent $NaHCO_3$, 0.005 per cent $NaH_2PO_4 \cdot 2H_2O$ and 0.05 per cent glucose to which was added 1 mM $MgCl_2$ to avoid spontaneous contractions. The plastic chamber was submerged at 30° C in a constant temperature water bath and was perfused with small bubbles of atmospheric air.

The sensitivity of the uterus to angiotensin could be increased by intramuscular administration of 0.01 mg of Khanyl (Dienoestrol acetate) or 0.001 mg of Ovex (oestradiol benzoate) to the rats 1–2 days before they were killed.

A biological 4-point assay with 2 dose levels of standard (CIBA val-angiotensin II amide 100 ng/ml in a solution of physiological saline) and 2 dose levels of unknown test solution was used. Each dose was administered to the chamber 4 times in 4 randomized series and an assay of the activity in a sample of the incubation mixture thus consisted of 16 solitary contractions. After each contraction the plastic chamber was rinsed several times from the bottom with the perfusion fluid and 2–3 minutes were allowed to elapse before the next volume was administered.

The Substrate Angiotensin I

This was prepared by incubating renin with angiotensinogen under conditions at which the converting enzyme was inhibited. According to *Skeggs* (1954) the enzyme has chloride ions as activator. In order to remove these dialysis was performed of renin (Hog Renin Nutr. Bioch. Corp., Cleveland) and of the angiotensinogen containing substrate—which consisted of heparinized plasma from 24 hour nephrectomized rats which according to *Bing* (1964) have a high content of angiotensinogen. The dialysis was performed at 4° C in Visking 24/32 dialysis bags against several changes of distilled water until the conductance measurement was the same as that of distilled water.

Thereafter renin was incubated in the ratio 3 Goldblatt units per ml plasma for 10 minutes at pH 5.8 and 37° C, since *Helmer* (1957) has demonstrated that the converting activity is very low at a pH below 6.

The pH was then brought to 5.3 with 2 M H_3PO_4 and after 10 minutes boiling in a water bath and centrifugation the pH of the supernatant was brought to 7.4 with 5 N NaOH and 3 ml portions of the clear solution of A I were stored at —20° C in siliconized tubes.

The A I formed in this way was also measured by a biological 4-point assay against CIBA val-angiotensin II amide by its action upon the blood pressure of rats anaesthetized with amytal and pretreated with ansolysen. The accuracy of the 4-point assay in measuring A II action on the uterus and blood pressure may be seen from Table 1 in which the action of 4 CIBA val-angiotensin II amide solutions with concentrations unknown to the investigator was tested against standard A II on the uterus and blood pressure.

TABLE 1

The Accuracy of 4–Point Assay of Angiotensin val II Amide on Rat Bloodpressure and Rat Uterus Calculated (Left) and Estimated Values (Right)

	Calculated A II conc in ng/ml	Estimated A II conc in ng/ml
Rat bloodpressure	69	71
	33	38
	119	109
	100	105
Rat uterus	84	82
	39	36
	136	134
	63	58

On the basis of 3 determinations of its action the quantity of the biologically formed A I was found to correspond to 433 ng/ml A II while its action upon the uterus compared with val5 A II amide corresponded to 48 ng/ml A II The effect of A I upon the uterus was thus only 11 per cent of its effect upon the blood pressure in rats where it was recorded as A II as A I is believed to be totally converted into A II on being injected into the blood stream of normal rats

Similarly *Cross & Turrian* (1961) found the oxytocic activity of synthetic A I to be 10 per cent of that of A II while *Page* (1961) found an effect of only 2–3 per cent thus the biologically A I in the substrate used in the present study may have been contaminated by small quantities of the octapeptide According to *Page* (1961) the action upon the uterus of AI is due to an inherent effect of the substance not to the conversion of A I to A II as a uterine extract proved to be devoid of converting activity

Plasma

It is known that plasma contains several constituents which can affect the rat uterus To avoid this plasma was dialysed for 24 hours at 4 C (Visking 24/32) in the quantities 1 ml of *heparinized plasma against* 500 ml of 0 9 per cent Na Cl to which had been added 10 ml of phosphate buffer with a pH of 7 4 The degree of dilution of the plasma at the dialysis was checked by weighing the dialysis bag at the commencement and conclusion of the dialysis On the average there was an increase in weight by 15 per cent subject to slight variations for which no corrections were made To make sure that the plasma did not contain substances which cause uterine contraction each plasma sample was tested on the uterus diluted with physiological saline and buffer in the same ratio but without addition of A I An inherent effect of the plasma following dialysis was observed in only one case

RESULTS

The study included investigation of (1) the converting activity in 9 normotensive human subjects with normal hepatic function compared with the converting activity in 9 cirrhotic patients with various degree of liver damage and (2) the converting activity in 9 normal rats and in 5 rats with ligated common bile duct

1 *Plasma from Human Subjects*

Fig 1 illustrates the conversion of A I into A II in the standard incubation system but at varying times using normal human plasma It is

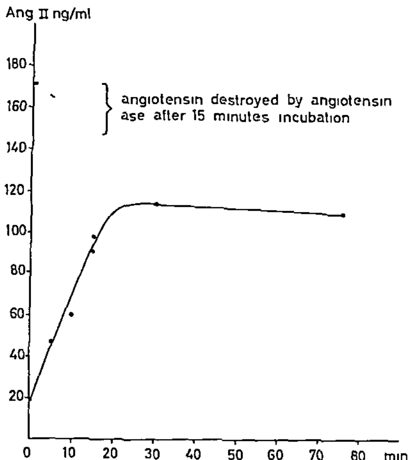


Fig 1

The influence of time on the conversion of Angiotensin I into Angiotensin II by incubating normal human plasma in the dilution 1/10 with Angiotensin I in the concentration 171 ng/ml 30 C pH 7 4

seen that the 50 per cent conversion corresponds to about 95 ng/ml and that the conversion reaches a level after which the A II content falls due to the angiotensinase activity In all plasma samples from human subjects as well as from rats with and without liver damage this activity was found to correspond to a destruction of between 10 and 15 per cent of the total quantity of angiotensin at an incubation period of 15 minutes

Fig 2 shows the conversion of A I to A II at various dilutions of the same plasma The incubation time was 15 minutes

Table 2 sets out the converting activity in 5 *normotensive persons* with normal hepatic function In the cases where the converting activity (expressed as the formed quantity of A II in ng/ml incubation volume) exceeded 130 ng/ml a control sample with the plasma dilution 1 *20* was prepared in order to titrate at values close to 50 per cent conversion

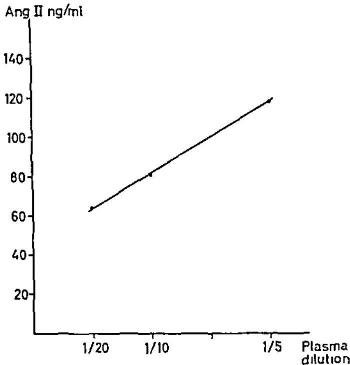


Fig 2

The conversion of Angiotensin I into Angiotensin II with different plasma dilutions at 30 C and the incubation time 15 minutes pH 7 4 Normal human plasma

TABLE 2

Plasma from Normal and Cirrhotic Patients The Values Are in Nanograms Angiotensin II/ml the Incubation Time 15 minutes 30 C pH 7 4

	Plasma sample	Plasmadilution 1/10	1/20
Normal human plasma	1	79 (78 81)	
	2	140	96
	3	95 (98 91)	
	4	137	110
	5	77	
Cirrhotic human plasma	1	95 (87 03)	
		146	96
	3	11	
		91	
		111 (110 112)	

Table 2 gives the [illegible] ing [illegible] in [illegible] *patients with hepatic cirrhosis* In this tab[illegible] specimens [illegible] plasma are arranged so that the specimen from [illegible] patient with the mildest degree of [illegible]ver damage is No 1 This patient had moderate inactive alcoholic cirrhosis without

any alteration of the protein fractions The degree of liver damage increases up to No 5 a clinically severe active cryptogenic cirrhosis with altered protein fractions and with histological signs of severe cirrhosis in an active phase

2 *Plasma from Rats*

Table 3 shows the converting activity in 5 female albino rats weighing 225–325 g Table 3 gives also the values for 5 rats in which the common bile duct had been ligated and cut by laparotomy under ether anaesthesia 8 days before the blood samples were taken It was found that on the average the rats could survive for 8 days after the procedure During this period they became greatly debilitated and deeply jaundiced In all cases autopsy showed severe jaundice with hepatic and biliary stasis

TABLE 3

Plasma from Normal Albino Rats and from Rats with Ligated Common Duct The Values Are in Nanograms Angiotensin II/ml the Incubation Time 15 Minutes 30 C pH 7 4

	Sample	Plasmadilution 1/10
Normal rat plasma	1	103
	2	82
	3	95
	4	92
	5	95
Plasma from rats with hepatic injury	1	119
	2	121
	3	100
	4	104
	5	76

DISCUSSION

The rats uterus proved to be an accurate and sensitive organ for testing A II formed from A I in incubation systems using rat and human plasma

Provided that impaired liver function entails a reduced converting activity the 5 cirrhotic patients would be expected to show less activity than the normal persons and the activity would be decreasing from No 1 to No 5 As is apparent from Table 2 this is not so as the values of the normal persons ranged from 77 to 140 ng/ml with an average of 109 ng/ml while those of the cirrhotic patients ranged from 91 to 146 ng/ml average 119 ng/ml

Comparison of the two groups of rats in Table 3 showed that the average conversion in the normal rats was 91 ng/ml while in the rats with hepatic damage it was 104 ng/ml

Accordingly it may be concluded that neither human subjects nor rats showed any signs suggesting that impaired hepatic function should entail a reduced converting activity On the contrary there was a slight increase in the converting activity in the presence of liver damage On the other hand it cannot be ruled out that the reduced converting activity which *Loyke* found in carbon tetrachloride poisoning may be due to a specific effect of this substance

SUMMARY

A method by which to determine the activity of the converting enzyme is described The converting enzyme in plasma was incubated with its substrate angiotensin I and the degree of conversion was measured on an isolated rat uterus By this technique it was found that an impaired hepatic function in human subjects and rats did not reduce the converting activity

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MESENCHYMAL TUMOURS OF THE STOMACH

A Histopathological Classification of 176 Tumours and a Follow-up Study of 160 Cases

By

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Too little is known of mesenchymal tumours of the stomach. A review of the literature shows a great variation of nomenclature. Some of the authors use terms such as "spindle cell sarcoma", "mixed cell sarcoma", "Rundzellsarkom" and "malignant haemangioendothelioma", others do not (*Marshall & Meissner* 1950, *Stout* 1953, *Giberson et al.* 1954, *Ro* 1961, *Trompke & Gregl* 1962). The criteria for classification also varies (*Golden & Stout* 1941, *Lemon & Broders* 1942, *Giberson et al.* 1954, *Trimble & Harkins* 1960, *Berg & McNeer* 1960, *Garvie* 1965). As a result the literature on the subject is confusing and data concerning biological behavior, curability, etc., are difficult to compare. Thus the authors' attention was focused on the subject and they were induced to perform this clinicopathological study, of which the histopathological classification is the first part. The clinical course and its correlation with the histopathological classification is presented in the second part of the study.

MATERIAL AND METHODS

The material consisted of 176 cases collected from the files of the Finnish Cancer Registry, comprising all cases diagnosed as malignant mesenchymal tumours and unclassified malignant tumours. Tumours diagnosed as benign mesenchymal tumours, and malignant tumours not reported to the Finnish Cancer Registry were collected from the files of all the pathological laboratories in Finland.[1]

Almost all cases were tumours removed by surgery, and covered the years 1953-1960. The material was thought to cover the majority of specimens sent for pathological examination during the period in Finland.

The slides were stained with haematoxylin-Van Gieson, haematoxylin-eosin

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1 The authors would like to express their gratitude for the co-operation received from the following institutes: Department of Pathology I, II and III, University of Helsinki, Department of Forensic Medicine, University of Helsinki, Department of Pathology, University of Turku, The Radiotherapy Clinic of University Central Hospital, Helsinki, Women's Clinic of University Central Hospital, Helsinki, Kivela Hesperia Hospital, The City of Helsinki Mehilainen Hospital, Helsinki, Central Hospital Kuopio and Central Hospital Tampere.

Mallory's phosphotungstic acid haematoxylin, Masson's trichrome stain and Gomori's silver impregnation stain for reticulin.

The slides were re-examined by one of the authors (G.T.) and classified according to histopathological criteria indicated below.

Following the histopathological classification inquiries were sent in May 1966 to the regional registry offices to obtain information whether the subjects were alive and, if not, to be informed of the cause of death.

Hospital records were complete for 174 cases. The 5-year follow-up material (Table 1) included 160 individuals whose histories were available.

A HISTOPATHOLOGICAL CLASSIFICATION OF 176 TUMOURS

The variation of classification convinced the authors of the necessity of choosing a widely known and authorized study as a reference base. The histopathological classification of the present material was mainly based on the classification and descriptions given by *Stout* in Atlas of Tumor Pathology (1953) with slight modifications. The present material comprised some tumours closely corresponding to a description given by *Martin et al.* (1960) and *Stout* (1962) of a type of tumour named by the latter "bizarre leiomyoblastoma". These required a separate heading, as did lesions called "benign lymphoid hyperplasia" (*Faris & Saltzstein* 1964). Six cases in which material for diagnostic purposes was insufficient were discarded. 17 tumours considered as carcinomas on grounds indicated below were also discarded, as were cases diagnosed as lymphatic leukaemia. The remaining material consisted of 176 tumours found in 174 patients. The classification is given below.

Leiomyoma		92
Ordinary leiomyoma	59	
Cellular leiomyoma	25	
Leiomyoma with pigmentation	8	
	92	
Bizarre leiomyoblastoma		5
Neurinoma		3
Benign lymphoid hyperplasia		3
Fibroma		2
Lipoma		4
Inflammatory polyp		4
Lymphosarcoma		35
Mb. Hodgkin		-
Leiomyosarcoma		20
Unclassified malignant		7
		176

In the following the tumours found are listed with a description of the corresponding histological picture.

Leiomyomas (92). The tumours showed varying degrees of differentiation towards smooth muscle tissue with interlacing bundles of spindleshaped cells. The tumour tissue often merged into surrounding smooth muscle tissue. Many tumours showed a high content of collagen. In a few cases Masson's trichrome stain showed red muscle fibrils. The

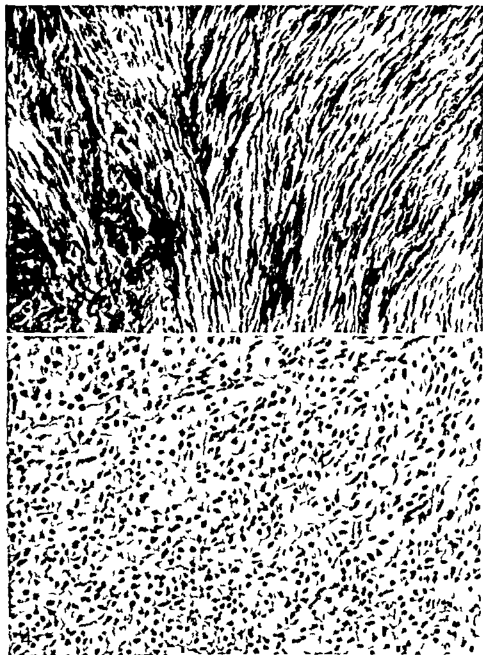


Fig 3 Cellular leiomyoma
Fig 4 Bizarre leiomyoblastoma

cuolated low cellular Antoni type B tissue and true encapsulation were considered as criteria for this label Tumours that were not encapsulated were as indicated above called leiomyomas although they showed regimentation of the nuclei which is considered to be characteristic of neurinomas

Inflammatory polyp (4) In these there was a proliferation of connective tissue cells in lamina propria and submucosa with oedema hyperaemia and a varying number of inflammatory cells mostly eosinophiles

Benign lymphoid hyperplasia (3) This lesion showed an abundance of mature lymphoid cells similar to those of lymphosarcoma Visible germinal centres an apparent intermingling of inflammatory cells or evidence of longstanding ulceration i e fibrosis around ulceration were according to *Faris & Saltzstein* (1964) considered as criteria of this type—One of these tumours was associated with an ordinary leiomyoma

Lipoma (4) These consisted of an accumulation of mature fat tissue in the submucosa

Fibroma (2) This type consisted of a mass of connective tissue without any traces of muscle fibres

Lymphosarcoma (35) These tumours showed abundance of more or less mature lymphoid cells and the absence of germinal centres The reticulum showed a varying dominance of large pale staining rounded or polygonal cells with large pleomorphic nuclei Mitoses were numerous In many cases the tumour showed a delicate reticulin network surrounding single cells The differential diagnosis towards anaplastic carcinoma was mainly based on negative grounds Tumours with row formation abortive glandular structures PAS positively staining mucin or a visible connective tissue stroma were called carcinomas and were discarded Neither of these criteria is clear and tumours where no decision could be made were called malignant tumours of unknown histogenesis One of these tumours was associated with an ordinary leiomyoma

Hodgkin s disease (2) Here the tumour was dominated by lymphoid cells and reticular cells Reed Sternberg s giant cells were found in every case

Leiomyosarcoma (20) In this group the microscopic pictures were often similar as among leiomyomas though all tumours considered leiomyosarcomas showed a very high cellularity The criteria for considering a tumour a leiomyosarcoma was an elevated mitotic rate or haemorrhages a ... e necroses of the tumour tissue Due to the method of collection of the material it was impossible to evaluate infiltration ifany ... the stomach wall which lessens the accuracy of the diagnosis in the present study

Malignant tumours of unknown histogenesis (7) These tumours showed clear microscopic signs of malignancy cellularity pleomor

phism high mitotic rate infiltrative growth However they lacked the above mentioned criteria of malignant mesenchymal tumours and those of carcinoma, and hence a histogenetic diagnosis of any accuracy could not be stamped upon them

Comments

The general distribution of the material among different tumour types corresponds roughly to that reported by *Stout* (1953) Some differences are though noted

The relatively greater number of leiomyomas and the smaller number of leiomyosarcomas might depend partly on the fact that the diagnoses were made almost solely on histopathological grounds in the present material This might imply that some cases looking histologically benign might show *e g* extraventricular growth A greater number of carefully selected blocks for microscopical examination might have revealed areas with high mitotic rates and indication of malignancy in tumours diagnosed in the series as benign Some leiomyosarcomas might also be found among the cellular leiomyomas

The relatively smaller number of lymphosarcomas in the material compared with Stout's is less surprising The distinction between *these tumours and the anaplastic carcinomas is by no means clearcut* and possibly some of the unclassified malignant tumours might even be called reticulum cell sarcomas

In general Stout's classification seemed practicable The low incidence of benign and lack of malignant neurogen tumours is surprising Stout pointed out that many tumours discussed in the literature and reported as neurinomas actually are leiomyomas The group leiomyomas with regimentation may possibly contain neurogenous tumours *e g* Schwannomas Though this group is structurally homogenous and many of the lesions included showed indisputably a gradual change into the surrounding smooth muscle tissue the authors consider this feature a strong indication of muscular origin

As a general conclusion this paper shows how much the classification *of tumours is a matter of agreement on criteria Further it stresses* the known fact that a pathological diagnosis should be built up on a broader base than that available from a few microscopic slides

EVALUATION OF THE HISTOLOGICAL RE-EXAMINATION OF 176 CASES

The distribution of all 176 cases by sex and age can be seen in Table 1 There was male predominance in the group of lymphosarcomas The 5 year survival rate is given in the same table

Ordinary leiomyoma The 5 year survival rate for cases of ordinary leiomyoma and cellular leiomyoma is about the same Thirty nine (79 6

per cent) out of the 49 cases of ordinary leiomyoma followed up were alive after five years It was not possible to demonstrate among the ten fatalities a single case in which the histopathological interpretation of the tumour conflicted with the cause of death

TABLE 1
Sex Age and Follow up of the Material

Tumours	Sex female	Sex male	Age mean	Age range years	Number of cases followed up		Living after 5 years
Leiomyoma	44	48	59 5	29–79	79		65 (82 3%)
Ordinary leiomyoma	26	33	59 6	29–79		49	39 (79 6%)
Cellular leiomyoma	12	13	55 3	35–79		24	20 (83 3)
Leiomyoma with regimentation	6	2	59 2	44–79		6	6
Bizarre leiomyoblastoma	3	2	50 2	26–71	5		4
Neurinoma	2	0	65 0	64–66	2		2
Fibroma	2	0	54 5	52–57	2		2
Lipoma	2	2	48 3	33–61	4		4
Inflammatory polyp	1	3	62 5	58–68	3		2
Benign lymphoid hyperplasia	0	3	48 3	38–64	3		1
Lymphosarcoma	13	24	56 6	25–77	36		9 (25 0%)
Leiomyosarcoma	12	8	54 3	31–73	20		7 (35 0%)
Malignant unclassified tumours	6	1	48 4	21–63	6		0
	85	91			160		

In one case however a malignant tumour of the stomach was suggested as the cause of death This can be considered as a mistake originating in the pre operative diagnosis of carcinoma of the stomach The patient aged 65 and in poor condition with severe cardiopulmonal disease had met for a check up three months before death there was no recurrence A suberous myoma the size of a little fingertip on the minor curvature had been excised five months earlier but no other specific pathological changes were found in the abdominal cavity or at gastrotomy No autopsy was performed Re examination of the microscopical slides did not change the diagnosis of a simple ordinary leiomyoma [illegible] perative deaths among patients with ordinary leiomyoma [illegible] oronary [illegible] lusion the other from acute renal insufficiency The [illegible] en death were caused by other diseases unconnected with the tumour [illegible] of them were cardiovascular affections

Cellular leiomyoma Twenty (83 3 per cent) out of the 24 cases followed up were alive after five years In one case there was a conflict between the histological interpretation of the tumour and the cause of death

The patient was reported to have succumbed to a malignant neoplasm of the stomach half a year after a radical resection But re examination of the microscopical slide did not change the diagnosis of cellular leiomyoma Only few mitoses were detected At the operation a well demarcated tumour the size of a hen's egg was found to be located in the minor curvature no metastases were detected Among the other fatal cases a woman aged 79 died of massive haemorrhage from the tumour

the cause was not found until at autopsy. The third death occurred as a consequence of surgical complications. Anaemia of unknown aetiology was entered as the cause of death in the fourth case. Operation was performed on the patient on account of ulcer of the stomach and the pea sized subserous myoma was only an incidental finding. By that time the patient had already ambiguous anaemia not caused by the bleeding ulcer. Death followed three months after resection of the stomach and could obviously not be attributed to the removed myoma. No autopsy was performed.

Bizarre leiomyoblastoma. Among the patients with bizarre leiomyoblastomas one was lost from complications three weeks after resection of the stomach. The other four were alive. All these cases will be described in detail in a later paper.

Leiomyoma with pigmentation, neurinoma, fibroma and lipoma. None of the patients with pigmented leiomyomas had died during the 5 year period. The same applies to patients in the neurinoma, fibroma and lipoma groups.

Inflammatory polyp. One man with a polypous mucosal tumour and a histological diagnosis of inflammatory polyp managed by excision through gastrotomy died four months after the operation according to the death certificate of a malignant tumour of the stomach.

Besides excision of the polyp, gastro-enterostomy and entero-anastomy were performed for a stenosing pylorus; firm paravertebral lymph nodes had also been palpated. Re-examination of the microscopical slides did not lead to a change of the diagnosis. However, no biopsy specimen was taken from the pyloric region or the lymph nodes. No autopsy was performed but apparently the patient did not die because of the inflammatory polyp that was removed. The other two cases of inflammatory polyp were alive.

Benign lymphoid hyperplasia. Among patients in the group of benign lymphoid hyperplasia one died of lung metastases two years after laparotomy performed on the basis of a clinical diagnosis of cancer of the stomach. Another succumbed to metastases to the liver 3½ years after total gastrectomy performed on the basis of a clinical diagnosis of a presence of three ulcerative carcinomas of the stomach. These two cases are analysed in more detail under the heading comments. The third patient is alive.

Lymphosarcoma, leiomyosarcoma and malignant unclassified tumours. Among the histologically malignant tumours the prognosis in cases of lymphosarcomas is worse than that in cases of leiomyosarcomas. Nine out of 36 (25.0 per cent) patients in the former group were still alive while in the cases of leiomyosarcoma seven out of 20 (35.0 per cent) patients were alive after five years. All six subjects with malignant unclassified tumours had died because of their neoplasm. One patient with lymphosarcoma of the stomach treated with subtotal gastrectomy as a palliative measure was still alive nine years after the operation. Another died of his neoplasm six years after laparotomy and biopsy. There were two postoperative deaths among patients in the lymphosarcoma group and three among those in the leiomyosarcoma group. In addition in one case of leiomyosarcoma

death was caused by a coronary occlusion 4½ years after excision of the tumour any recurrence of the neoplasm was not observable One patient with lymphosarcoma and a concomitant hearth disease died 4½ years after radical resection The remaining 24 deaths from lymphosarcoma and nine from leiomyosarcoma were caused by the malignant tumour

Comments

Lymphosarcoma Among the cases of lymphosarcoma correlation between the first diagnosis and the diagnosis established after re examination was relatively good The first diagnosis had been undetermined malignant tumour in two cases carcinoma anaplasticum in one instance and polymorphocellular sarcoma in three cases One patient with lymphosarcoma is alive nine years after palliative subtotal gastrectomy Re-examination of the microscopical slides disclosed a considerable number of plasma cells in the lymphoid masses This together with the peculiar distribution of the lymphoid masses could motivate a change in the malignant diagnosis to benign lymphoid hyperplasia However the operative status was characteristic of a malignant tumour of the stomach A great tumour extending 5 cm from the cardia to 10 cm from the pylorus was found Lymph nodes were also palpable paravertebrally

Another patient with lymphosarcoma lived for six years after laparotomy and biopsy Re examination of the slides showed only a uniform solid mass of fairly mature lymphocytes The criteria for benign lymphoid hyperplasia could not be found and thus the diagnosis of lymphosarcoma could not reasonable be changed The fact that patients with lymphosarcoma of the stomach survive for fairly long periods of time in spite of incomplete therapy has been observed by *Allen et al* (1954) *Friedman* (1959) *Ackerman & Butcher* (1964) *Trompke & Gregl* (1962) and others

Benign lymphoid hyperplasia All three cases of benign lymphoid hyperplasia had earlier been interpreted as sarcomas and in the light of the follow up this seems justified A diagnosis of benign lymphoid hyperplasia should obviously [illegible] with caution

One patient with benign lymphoid hyperplasia died two years post operatively presenting pulmonary [illegible] The patient was a 38 year old male Laparotomy revealed an inoperable tumour about 6 cm in diameter with [illegible] in the lesser curvature Firm paraaortal lymph nodes were [illegible] palpable Microscopy showed a massive proliferation of lymphoid cells partly infiltrating the muscular wall The diagnosis of benign lymphoid hyperplasia was based upon many visible fibrosis as evidence of long standing ulceration Re examination however disclosed atypia and pleomorphism of the lymphoid cells [illegible] was primarily explained as a co-existent acute nonspecific

inflammation At re-examination the atypia was considered to be too marked to suggest a benign diagnosis and the latter was corrected to lymphosarcoma

Another patient with benign lymphoid hyperplasia died 3½ years postoperatively of liver metastases according to the death certificate At operation one tumour about 2 cm in diameter was found in the middle of the lesser curvature another of the same size was located to the cardiac region and a third was observed in the prepyloric region all containing ulcers Total gastrectomy was performed On the minor curvature near the tumour there were numerous lymph nodes The histological diagnosis of benign lymphoid hyperplasia was based on the presence of numerous lymphoid follicles visible in the stomach wall Re-examination of the slides revealed follicles of considerable size mostly surrounded by a ring of mature lymphocytes sometimes changing into solid masses of lymphoid cells infiltrating all layers of the stomach wall Re-examination thus justified a correction of the diagnosis into one of giant follicular lymphosarcoma This underlines the observation by Ackerman and others that existence of lymphoid follicles does not exclude a diagnosis of lymphosarcoma if the microscopical finding as a whole indicates malignancy The need to study a great number of microscopical slides especially when lymphoid tumours of the stomach are involved is also emphasized by this apparently false diagnosis

TABLE 2

Earlier Histopathological Diagnoses of Leiomyosarcomas and 5 Year Survival Rates

EAD	Number of cases	Living after 5 year
Leiomyoma	*3*	*2*
Leiomyosarcoma	7	3
Myoma malignum	2	1
Sarcoma fusocellulare	3	0
Sarcoma neurogeneum	1	1
Fibrosarcoma	1	0
Tumor malignus	3	0
	17	5
Total	20	7

Smooth muscle tumours In most cases differences from the earlier interpretation were noted among the cases of smooth muscle tumour Leaving aside the question of whether the tumour is of myogenic or neurogenic origin it is noteworthy that the leiomyosarcoma group included three patients in whom tumours earlier had been diagnosed as benign leiomyomas of these two were alive after five years (Table 2) On the other hand in the group comprising 24 cases of benign cellular leiomyomas seven lesions had earlier been classified as leio

myosarcomas and seven as other types of malignant fusocellular sarcomas. Among these twelve (85.7 per cent) were still alive at the time of the follow up (Table 3).

TABLE 3

Earlier Histopathological Diagnosis of Cellular Leiomyomas and 5 Year Survival Rates

PAD	Number of cases	Living after 5 year
Leiomyoma	7	6
Neurinoma	1	0
Fibroma	1	1
Neuroblastoma	1	1
	10	8
Leiomyosarcoma	7	7
Myoma malignum	3	2
Sarcoma fusocellulare	1	1
Neurinoma malignum	2	2
Neurosarcoma	1	0
	14	12
Total	24	20

One patient with cellular leiomyoma died six months after a radical hemiresection of the stomach according to the death certificate of a malignant neoplasm of the stomach. This may imply that a histologically benign cellular leiomyoma may behave as a malignant tumour as noted by *Ackerman & DelRegato* (1962) and others. Or it may imply that a patient once given a diagnosis of malignancy is given the same diagnosis on his death certificate although death might have been unrelated to the tumour. In this case the earlier diagnosis was neurosarcoma.

All the bizarre leiomyoblastomas were earlier diagnosed as malignant myogenic or neurogenic tumours.

The results of the follow up of cases of malignant tumours are in support of the general opinion that sarcomas of the stomach have a more favourable prognosis than carcinoma. This in spite of the fact that many tumours previously diagnosed as fusocellular sarcomas in our classification were considered benign. *Jordan et al* (1955) gave a 5 year survival rate of 42.6 per cent for lymphosarcomas and 51 per cent for leiomyosarcomas among patients treated radically. Nevertheless including laparotomies and palliative procedures the 5 year survival rate seems to be equal to or better than the rate after radical treatment of carcinoma.

SUMMARY AND CONCLUSIONS

In a material of 176 mesenchymal tumours of the stomach re-examined and classified histologically 160 cases could be followed for five years

The histological diagnosis was assessed. Care should be exercised in diagnosing benign lymphoid hyperplasia; the clinical picture and the operative status must be taken into consideration. In the group of cellular leiomyomas more than one half of the cases had earlier been diagnosed as malignant fusocellular tumours, a finding that did not seem to be justified in the light of the follow up. Otherwise the results were in support of the general opinion that sarcomas of the stomach have a better prognosis than carcinomas.

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INCORPORATION OF THE ^{3}H THYMIDINE IN THE EPITHELIAL CELLS OF THE GASTROINTESTINAL TRACT OF HEPARINIZED MICE

By

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Heparinization of rats was found to cause a sharp decrease in mitoses in the gastric epithelium but not in the epithelium of intestinal mucosa (*Rasanen* 1963 *Rasanen Cederberg & Taskinen* 1966) The DNA synthesis is inhibited by the effect of glucocorticoids especially in the gastric mucosa (*Lahtiharju Rasanen & Teir* 1964) The number of mitoses in the gastric mucosa declines when mast cells become degranulated under the influence of glucocorticoids which probably release local heparin (*Rasanen* 1963) When gastric mucosa under the action of ACTH has totally lost the metachromatic material of its mast cells (*Rasanen* 1961) the number of mitoses is trebled in the epithelial cells (*Teir & Rasanen* 1961)

Heparin has been found to have a reversible inhibitory effect on growth in cell cultures (*Fisher* 1936 *Heilbrunn & Wilson* 1949 *Csaba et al* 1961) and continuous heparinization decreases the number of lung metastases caused by the application of tumour cell suspension in rats (*El Rifi et al* 1965)

Retardation of regeneration may be caused by the inhibition of the DNA synthesis in the epithelial cells of the gastrointestinal canal due to heparinization This phenomenon is the object of the present study where regeneration is determined autoradiographically following the injection of ^{3}H thymidine

MATERIAL AND METHODS

The animals used for the study [illegible] white male mice aged about 2 months of a non homologous strain They were allowed standard commercial food and water *ad libitum* throughout the period of the experiment

Group I 6 mice mean weight [illegible]4 g received 1 × 50 u [illegible] f heparin (Medica) in 0 1 ml of saline intraperit[illegible] [illegible] 6 hours before sacrifice

Group II 6 mice mean [illegible]ght 24 g r[illegible] d 3 × 50 u [illegible] of heparin at 1 hour intervals The last inje[illegible] [illegible] given in th[illegible] morning at 09 0 hours [illegible] hour before sacrifice

The study was aided [illegible] ants from [illegible] [illegible] Juselius Foundation [illegible] nd *The Finnish Research Coun[illegible] f Medic[illegible]*

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PNEUMATOSIS INTESTINALIS

A Pathogenetic Study

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Pneumatosis intestinalis also called pneumatosis cystoides intestinalis gas cysts of the gut or pneumocystis intestinalis is a rare condition characterized by gas filled blisters in the submucous and/or subserous surfaces of the small intestine and colon The ventricle with suspending structures and the mesentery sometimes are involved The disease occurs most frequently in males between the ages of 30 and 50 and is symptom free or accompanied by a wide range of digestive and abdominal disturbances Spontaneous resolution has been assumed A substantial divergence of opinion prevails in the concept of aetiology and pathogenesis

On gross examination the honey comb like blisters have been seen predominantly on the serous side or on the mucous side In the description of the histology of the blisters the very characteristic histiocytes and giant cells in the inner lining have been repeatedly noted Many authors presume primarily lymphatic involvement and pretend that the cells mentioned are derivatives of endothelial cells *Koss* (8) described other inflammatory cells such as eosinophilic granulocytes lymphocytes and plasmocytes in the tissue reaction that during the ageing of a single lesion evolves more and more granulomatous character and eventually causes obliteration of a single blister No fat or other abnormal metabolic products have been found in the lining cells

Approximately 250 cases are known in the literature In the majority of cases the gas cysts have been found in association with other diseases and traumas to the gastrointestinal tract Conditions leading to pyloric obstruction have been found in more than half of the cases This type has been called secondary pneumatosis in contrast to the primary pneumatosis with unknown aetiology Recently attention has been drawn to pulmonary pathology and it has been claimed that there is no primary pneumatosis (7) Various theories of the evolution of the gas cysts have been postulated a comprehensive review is included in *Koss* article (8) in 1952 Up to now the opinions about the mechanism involved in cyst formation can be divided into those of a mechani



Fig 4

The upper field shows the stretched borderline of a distended early blister (arrow). There is a lot of elastic tissue in the outer wall. In the middle field a lateral pouch of a larger blister. Note the fibrous walls and the large basophilic giant cells. Unfortunately postmortal autolysis has destroyed the mucous epithelium.

could be traced into the cytoplasm of the histiocytes and giant cells The latter appeared to be the most active and formed often long ribbons along the inner surface of the blister Some of them were more rounded and were seen to drop off and float free in the cyst Some eosinophilic granulocytes and lymphocytes appeared early among the histiocytes but larger collections could be seen later in association with the more advanced fibrocellular proliferation of the cyst wall (Fig 5) Especially the slit like pouches in the lateral margins of the cysts were surrounded by this type of granulation tissue With increasing formation of collagen the cysts seemed to contract and were finally obliterated This was preceded by a pronounced increase of histiocytes and giant cells in the inner lining (Fig 6) An elongated patch of scar tissue surrounded by a curled and broad frame of elastic tissue was left behind Sometimes a thick walled slit persisted and the endothelial or histiocytic lining cells were almost absent (Fig 7) The scar tissue showed often many small tortuous vessels

UV and blue light fluorescence study of the unstained sections did not show any autofluorescent material in the cyst content of walls

COMMENT

The existence of the diverticula in the duodenum and ileum places this case to the group of secondary pneumatosis Although simple and innocent the diverticula may have had something to do with the cyst formation The theory of a mechanical action seems to be best applicable Gases in the bowel may have been trapped in the diverticula and forced through the mucosa to the tissue of the intestinal wall where they have been squeezed aborally by peristalsis There was nothing to substantiate the theories of a chemical deficiency or bacterial action as the origin of the gas Nor were there traumas pulmonary diseases or obstructive conditions to indicate other types of mechanical factors in the pathogenesis The location of the cysts in the submucous connective tissue and/or in the muscularis propria fits in with the assumption that the pathway of the gas had been from the inside of the bowel and along the loose submucous connective tissue to a more distally located segment of ileum

The earliest morphological changes in connection with the development of the cysts support the opinion that the gas accumulated initially inside the lymphatic vessels The evidence however was not conclusive The difficulty was to foresee whether one or the other of the slit like spaces resembling lymphatic vessels was going to be a cyst The factors responsible for the productive inflammatory reaction in the cyst wall must be at least two One is the mechanical stretching of the walls caused by the peristalsis the other refers to the apparent irritating action of the gas itself

The cellular reaction and the remarkable pericystic fibrosis must be

mutually dependent in the meaning that the histiocytes and giant cells account for the marked fibroplasia. In other words they are not only a manifestation of a possible foreign body reaction to the irritating influence directed against the walls of the primordial cyst but an effective structure in the production of fibrosis. This view is supported by the finding of the intimate relationship between the cells and the fibres and by the presence of the acid mucous substances usually connected with fibroplasia. The appearance of lipids in this connection remains obscure but they may also be related to the neoformation of the fibres. Participation of elastic fibres in this neoformation is particularly interesting. It certifies for the close relationship between the different types of connective tissue fibres.

SUMMARY

A case of Pneumatosis intestinalis is presented. The disease was found in the small gut as an additional finding at autopsy of a 70 year old farmer who died due to an intracerebral vascular accident. The upper gut showed three diverticula and the gas cysts were found distally in the submucosa and muscularis propria of the ileum. Microscopically the blister formation appeared to commence with slit like spaces resembling lymphatic vessels. They showed an inner lining of thin endothelial cells surrounded by a zone of dense and wavy collagen and a periphery sometimes rich in elastic fibres. The walls of older blisters showed a peculiar fibrocellular reaction and giant cells of foreign body type. This progressed to contraction and obliteration of the single cyst. It was thought according to the theory of a mechanical blister formation that the gas originating in the diverticula was forced through the mucosa and transported distally in the submucous layer. The histiocytes and the giant cells in the inner lining of the cyst contained lipids and mucopolysaccharides. These cells were considered to account for the remarkable fibroplasia in the cysts walls.

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CHANGES IN HeLa CELL ULTRASTRUCTURE UNDER CONDITIONS OF REDUCED GLUCOSE SUPPLY

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Most currently used nutritive media for the maintenance of mammalian cells *in vitro* whether supplemented with serum or not have a glucose content of 100 mg% (5 5 mM) which corresponds to a normal mammalian blood sugar level or more (*Waymouth* 1965)

It is generally agreed that cell cultures require relatively high levels of glucose which serves principally as a source of energy and also as a precursor for other substances—at least in minimal essential media (*Eagle* 1960 *Levintow & Eagle* 1961) Is has also been generally observed that under normal growth conditions aerobic glycolysis predominates over respiration as carbohydrate breakdown pathway (*Paul* 1965) However *Graff et al* (1965) have demonstrated the possibility of prolonged growth of suspension cultures of L 929 mouse fibroblasts and Ehrlich ascites cells in media with very low glucose concentrations (5 mg%) Under these conditions the cellular glycolysis as measured by lactic acid production was reduced to zero In a radiometric study of chick fibroblast metabolism *Broda et al* (1961) observed a decrease in the glycolysis/respiration ratio (corresponding to the Crabtree effect) as the cell number per volume unit of medium is increased

The carbohydrate metabolism of HeLa cells under conditions of varied glucose supply deviates from these patterns According to *Paul* (1965) HeLa cells subjected to glucose concentrations above that of normal media exhibit a demonstrable Crabtree effect The same effect has been noted by *Abdel Tawab et al* (1959) who on the other hand found it impossible by reductive variations in the glucose concentration of the medium to force the carbohydrate metabolism into predominantly oxidative pattern While the glycolytic rate of HeLa cells falls off with increasing number of cells—i e with decreasing amount of glucose available per cell—there is no corresponding compensatory increase of respiration (*Ruel et al* 1963) *Eagle et al* (1958) observed

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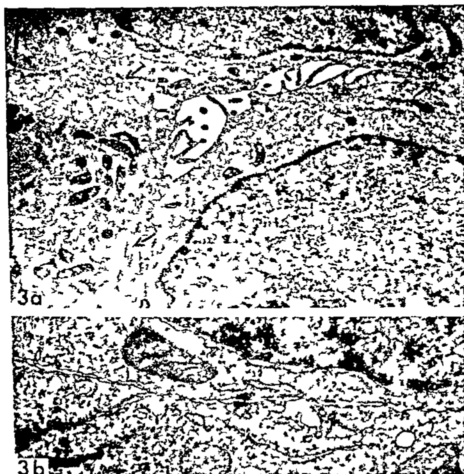


Fig 3 a and b

a Intercellular space at the junction of 3 cells experiment medium The extension of protrusion filled gaps is greatly reduced and the occurrence of smooth cell borders (arrows) is characteristic of the growth pattern 15 000 ×

b Part of uncomplicated cell border with desmosome experiment medium 20 000 ×

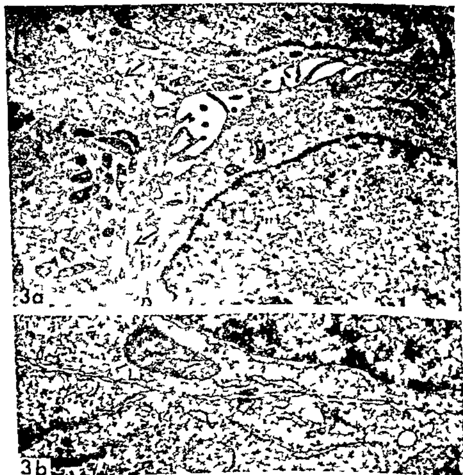
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Fig 1 Part of free periphe[illegible] f H[illegible] tanda d medium An [illegible] ate system of contorted c[illegible] t [illegible] on[illegible] nstitutes lagunae a[illegible] s in the peripheral e[illegible] 2 [illegible]

Fig 2 Intercellular space st[illegible] Th[illegible] ttern of growth i[illegible] characterized by rather wide inter[illegible]llula[illegible] ga[illegible] s [illegible] den[illegible] filled with [illegible] pl[illegible] mic protrusions 17 500 ×

1

2



Fi j 3 a an l b

a Intercellular space at the junctic n f 3 cells experiment medium The extension of protru ion filled gaps is greatly reduced and the oc urrence of smooth cell borders (arr)ws) is characteristic of the gr wth j attern 15 000 ×

b Part of uncomplicate l cell t rder with desm s me experiment medium 20 000 ×

F j t 1 2

Fig 1 Part of free (pl ery f Hela cell gr w tandard medium. An intricate system f c r d cyt pl mic r tri n tributes lagunae and slits in the periph r (yla m. 2 000 ×

Fig Intercellular tandar l me lium 1) rr f grewth is characterized l y rather wi t rcell ilar gap m r r nsely fille l with cytoplasmic protrus n 00 ×

is chemically different from the nutritive medium and possibly more favourable for the survival and functions of the cell Such a micromilieu might serve as a buffer zone between the cell and its surrounding medium

3 Bringing about cellular attachment and/or locomotion *i e* serving as pseudopodia rather than microvilli

The maintenance of a micromilieu necessitates a highly developed complex of surface convolutions and protrusions which constitute a layer covering the part of the cell surface exposed to the nutritive medium Electron microscopy of surface replica specimens of HeLa cells reveal that the development of cytoplasmic protrusions is far more complex and intricate along the rim of the cells than on their upper free surface and that the protrusions are in all locations tubiform or filiform and of rather constant diameter The flattened branched and straight extended form characteristic of the typical pseudopodia of migrating fibroblasts (*Willoch* unpublished) has not been observed Morphological considerations may point to an increase of the surface area as the most probable function of the surface protrusions Their cytoplasmic contents as seen in sections do not show differentiation features indicative of metabolic transport but the possibility can not be excluded that they contain a streaming cytoplasm or that there is a constantly changing process of retraction and generation of protrusions

Several features of the chemical composition of the culture medium influence the morphology and surface properties of cells cultivated *in vitro* (*Weiss* 1962 *Willmer* 1960) Variations in type and concentration of serum added to the medium may cause great alterations in outline and growth pattern of cells (*Taylor* 1962 *Willmer* 1960) Dissociated embryonic cells will not recombine effectively unless adequately supplied with glucose (*Moscona* 1962)

The cultures of the present study were supplied with fresh medium 3 times a week Reduction of the glucose content from 100 mg% to 6 mg% implies a change of about 5 mM in the total osmolal concentration initially offered to the cells at each medium renewal a change probably well within the limits of concentration changes exerted upon the medium by the cells own metabolic processes The concentration of electrically charged particles has not been changed Differences in surface morphology are probably not results of a direct influence of the environment on the chemical composition of the cell surface As the nutritive conditions have not been kept constant by a perfusion technique the cultures can not be subjected to quantitative studies of metabolism or other functions related to the composition of the medium The different development of cytoplasmic protrusions can only be viewed as morphological expressions of quantitative differences in glucose metabolism induced by changes in the cells glucose supply

As judged from the undiminished growth rate and healthy appearance of the HeLa cells under conditions of limited glucose supply it can be concluded that their basal glucose demand is small compared with the supply usually provided and with the quantities metabolized under normal culture conditions Under experimental conditions the cells are apparently fully capable of performing all vital functions including functions that can possibly be ascribed to the cytoplasmic protrusions The degree of development of such protrusions at any stage of a healthy growing cell is probably indicative of the cells need for protrusions and their functions As the HeLa cells demands are apparently covered at a glucose concentration of 6 mg% the high degree of development of cytoplasmic protrusions at standard conditions may be considered a secondary morphological feature illustrating the cellular reaction expressed as increase of surface area at increased glucose metabolism

The selectivity of cell contact and adhesion (*Moscona* 1965) is probably related to chemical structure of cell surface the establishment of adhesive contact between cells being dependent on equality correspondance or complementarity of their surfaces (*Steinberg* 1962) A high degree of diversity in surface structure corresponding to a wide distribution of different genetic constitution occurring in malignant tumours and in long term cell cultures may theoretically contribute to reduced adhesive contact An indicator of the degree of contact between cells is their separability by chelating agents such as versene (EDTA) Cells having a physiologically well established contact stabilized by the deposition of intercellular substances can not be effectively separated merely by a chelating agent (*Ambrose* 1966) In the present study the reaction of the cells to versene was tested both with cultures 3 days after passage and with cultures allowed to grow for one or two weeks undisturbed by intervening tryptic separation of the cells The uniformly high degree of separability of the HeLa cells showing a generally low adhesion indicates that limitation of glucose supply does not essentially alter the adhesive properties of the cells but results in a reduced development of surface protrusion with consequent smooth cell borders However the establishment of intercellular contact to the point of development of desmosomes as seen on cells grown in experiment medium probably demands an existing correspondence in surface structure The lack of contact between cells grown in standard medium is probably an expression of environmentally induced metabolic factors rather than intrinsic diversity of the cells

SUMMARY

An electron microscope study has been made of morphological features of HeLa cells growing in a standard nutritive medium containing 00 mg% glucose and in an experiment medium containing 6 mg% glucose

is chemically different from the nutritive medium and possibly more favourable for the survival and functions of the cell. Such a micromilieu might serve as a buffer zone between the cell and its surrounding medium.

3. Bringing about cellular attachment and/or locomotion, *i.e.* serving as pseudopodia rather than microvilli.

The maintenance of a micromilieu necessitates a highly developed complex of surface convolutions and protrusions which constitute a layer covering the part of the cell surface exposed to the nutritive medium. Electron microscopy of surface replica specimens of HeLa cells reveal that the development of cytoplasmic protrusions is far more complex and intricate along the rim of the cells than on their upper free surface and that the protrusions are in all locations tubiform or filiform and of rather constant diameter. The flattened branched and straight extended form characteristic of the typical pseudopodia of migrating fibroblasts (*Willoch*, unpublished) has not been observed. Morphological considerations may point to an increase of the surface area as the most probable function of the surface protrusions. Their cytoplasmic contents as seen in sections do not show differentiation features indicative of metabolic transport, but the possibility can not be excluded that they contain a streaming cytoplasm or that there is a constantly changing process of retraction and generation of protrusions.

Several features of the chemical composition of the culture medium influence the morphology and surface properties of cells cultivated *in vitro* (*Weiss* 1962, *Willmer* 1960). Variations in type and concentration of serum added to the medium may cause great alterations in outline and growth pattern of cells (*Taylor* 1962, *Willmer* 1960). Dissociated embryonic cells will not recombine effectively unless adequately supplied with glucose (*Moscona* 1962).

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As judged from the undiminished growth rate and healthy appearance of the HeLa cells under conditions of limited glucose supply it can be concluded that their basal glucose demand is small compared with the supply usually provided and with the quantities metabolized under normal culture conditions Under experimental conditions the cells are apparently fully capable of performing all vital functions including functions that can possibly be ascribed to the cytoplasmic protrusions The degree of development of such protrusions at any stage of a healthy growing cell is probably indicative of the cells need for protrusions and their functions As the HeLa cells demands are apparently covered at a glucose concentration of 6 mg% the high degree of development of cytoplasmic protrusions at standard conditions may be considered a secondary morphological feature illustrating the cellular reaction expressed as increase of surface area at increased glucose metabolism

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SUMMARY

An electron microscope study has been made of morphological features of HeLa cells growing in a standard nutritive medium containing 100 mg% glucose and in an experiment medium containing 6 mg % glucose

Cells grown in standard medium have a highly developed system of cytoplasmic protrusions extending into the medium at the free cell surface and constituting a complicated pattern of the contact zone of the cells which are thus rarely closely apposed to each other Surface protrusions of cells grown in experiment medium are less developed and smooth straight intercellular borders equipped with desmosomes are a common feature Surface replica specimens revealed no additional differences between cells from the two media

Disaggregation experiments with versene gave equal results with cells from the two media indicating that the differences of cell surface and intercellular spaces are of a purely morphological nature and do not imply differences in chemical properties of cellular contact

The degree of development of cytoplasmic protrusions was interpreted as indicative of the metabolic activity of the cells the total cell surface area increasing with increased glucose metabolism

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GONADOBLASTOMA (GONOCYTOMA III) IN A BOY WITH XO/XY MOSAICISM

Case Report with a Survey of Literature

By

KRZYSZTOF BOCZKOWSKI JERZY TETER HANNA TOMASZEWSKA and JOHN PHILIP

Received 5 1 67

The occurrence of a high percentage of neoplastic changes in dysgenetic or underdeveloped gonads is known from several publications (23 24 29 45 46 56) The etiology of these tumours is better understood from clinical and experimental work which has been published during the last few years (17 18 23 45 48 49 50 51) Most of gonadal tumours derive from germinal elements *e g* dysgerminoma (seminoma) embryonal carcinoma teratoma and chorioncarcinoma whereas tumours of Leydig (theca) cells and those of Sertoli granulosa cells represent respectively mesenchymal and sex cord derivatives

In 1953 *Scully* (41) described a new type of tumour build of germ cells Sertoli granulosa cells and interstitial Leydig cells Since then several new cases have been published It seems that comparison of these cases will give further information about the etiology of this tumour and also some clinical findings important in diagnosis and management

The main purpose of this study is to present a new case of gonadoblastoma (gonocytoma III – according to classification proposed by *Teter* (51)) and to compare the data of already published cases

CASE REPORT

The patient P W (Fig 1-3) aged 9 legal sex male has one older sister who is normal His mother received no hormonal treatment during pregnancy Delivery took place in the eighth month after three weeks of uterine bleeding At birth features of prematurity (weight 1650 g) and ambiguous external genitalia were noted

When the child was 8 5 years old his height was that of a child of 6 5 years His skeletal age was in accordance with chronological age Intellectual development was

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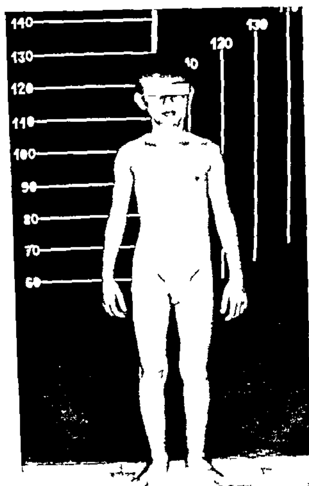


Fig 1

Case P W when 10 years old (height 1 7 cm)

normal (Echsler score) Congenital malformations of heart right kidney and ureter were found The external genitalia were ambiguous Small vaginal opening with a urethral meatus was present at the base of the phallus A bifid scrotal sac was empty Radiological studies (hystero alpingography) showed vagina and a small uterin cavity

Laparotomy showed a very small uterus and one Fallopian tube with small oval gonad situated in the place normally occupied by the right ovary Small testis with epididymis was found on the left The surgeon brought down the testis into the upper portion of the left side of the scrotal sac after taking a biopsy for microscopic study The uterus and right gonad were not operated upon and were left in their abdominal location

The microscopic study of the biopsy specimen from testis showed small dysgenetic seminiferous tubules lined by undifferentiated Sertoli cells and germinal cells Laminated eosinophilic intratubular bodies encircled by the inner layer of Sertoli granulosa cells were observed A radial arrangement of cells surrounding these intratubular bodies and resembling a corona radiata were the most striking feature (Fig 4) The outer layer of the gonad was composed of connective tissue resembling cortical ovarian like stroma



Figs 2–3

Fig 2 Case P W Ambiguous external genitalia Left testis was brought up at operation and fixed in the labioscrotal sac

Fig 3 Case P W Hysterosalpingography Vagina and rudimentary uterine cavity are visible

Examination of smears from buccal mucosa showed a negative sex chromatin pattern Cytogenetic analysis from blood (*Moorhead et al* 1963) and from skin (*Philip* 1967) showed 45/XO//46/XY mosaic Table 1

TABLE 1

Results of Chromosomal Counts in Case P W

No of chromosomes	<44	44	45	46	47	Total
Blood	2	1	19	10	–	3
Skin	1	3	14	12	–	30

Seven cells with 46 chromosomes and seven cells with 45 chromosomes were analysed The cells with 46 chromosomes uniformly had apparently an XY constitution whereas the constitution of those with 45 chromosomes was interpreted XO Three cells with 44 chromosomes were also analysed and showed no constant pattern We concluded that these were artefacts which probably arose during the making of the preparations possibly by heating

The results of blood groups analysed by Dr R R Race and Dr R Sanger are given in Table 2

TABLE 2

Data on Blood Groups in Family P W Analysed by Dr R R Race and Dr R Sanger

	ABO	MN	Rh	K	Xg	
P W	A_1	MN	R_1r			case P W
P S	O	MN	R_1r		+	father
P M	A_1	MN	R_1R_1			mother



Fig 4

Case P W Laminated eosinophilic intratubular bodies and dysgenetic tubules of left rudimentary testis

One year later the boy was once again admitted to the Clinic because of a pain and sudden symptoms of incarcerated inguinal hernia which protruded into the right scrotal sac The emergency surgical intervent on revealed in the hernial sac the uterus Fallopian tube and oval irregularly bossed gonad All these structures were removed

Histological examination of the gonad revealed two patterns

1 The cortical part was fibrotic partly sclerotic and hyalinized Within the stroma several small nests of germ cells scattered among sex cord cells (Sertoli granulosa type) were found (Fig 5)

2 In the vicinity of the above mentioned formations the typical neoplastic nests characterized by a confusing mixture of germ cells and sex cord cells in malignant fashion were found (Fig 6) Connective tissue which separated the neoplastic nests contained only a few spindle shaped cells and small groups of interstitial Leydig like cells In other part the neoplastic nests composed mainly of Sertoli granulosa cells were arranged in folliculoid pattern and contained calcified eosino philic bodies (Figs 7 and 8) This miniscule tumour was classified as gonadoblastoma (gonocytoma III)



Figs 2 3

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TABLF 4
Histo Pathological Data

References	Right gonad	Left gonad
41 case F C	Gonadoblastoma Small cells surround ing eosinophilic laminated bodies a few of which were calcified. Calcifications Fibrous stroma at the periphery	Not identified
41 case S T	Gonadoblastoma Area of pure dysgerm inoma lymphoid stroma and giant cells of Langhans type Calcifications Fibrous stroma at the periphery	Absent
52 53 56	Gonocytoma III Marked calcified concretions	Fibrous streak with bi sexual formations
22 23 case G K	Dysgenetic testicular tissue	Immature testicular tissue Gonadoblastoma Foci of seminoma calcifications
22 23 case J M	Seminiferous tubules lined mainly by Sertoli cells and a few spermatogonia	Gonadoblastoma Calcifications
2 38 44	Gonadoblastoma. Nest of dysgerminoma A few giant cells of Langhans type	Fibrous stroma
43	Testes with seminiferous tubules containing spermatogonies Gonadoblastoma	Fibrous streak with Call Exner bodies
15 16 case D L 1 (propositus)	*Gonadal streak fibrous stroma Nodule* gonadoblastoma Many cell nests con taining eosinophilic or calcified bodies Tumor solid teratoma containing smooth muscle (cartilage nerve tissues and gial elements	*Gonadal streak fibrous* stroma Nodule gonadoblastoma Many cell nests containing eosinophilic or calcified bodies
15 16 case D L. 2	Gonadal streak fibrous stroma Nodule gonadoblastoma findings identical with those in case D L. 1	Gonadoblastoma find ings identical with those in case D L. 1
33	Gonadoblastoma Absence of normal ovarian tissue	Gonadoblastoma Absence of normal ovarian tissue
53 57	Fibrous stroma Gonadoblastoma Marked calcified concretions	Fibrous streak with bi sexual formations (traces of gonadoblastoma)
13	—	Gonadoblastoma Small cells arranged circumferentially around an eosinophilic laminated material Calcifications

TABLE 4 (cont)

References	Right gonad	Left gonad
45	Gonadoblastoma Calcifications	Gonadoblastoma Calcifications
39 case 4	Infantile testis Gonadoblastoma Call Exner bodies Calcifications	Absent
55	Gonadoblastoma with large nests of dysgerminoma (seminoma) and syncytiotrophoblast cells	Fibrous stroma with bisexual formations Gonadoblastoma
40	Testis with gonadoblastoma Calcifications	Testis with gonadoblastoma Calcifications
30	Absent	Testis with gonadoblastoma Intra tubular egg Calcifications
58	Undescended testes Gonadoblastoma present in one of them	
54	Dysgenetic testis with gonadoblastoma in situ	Fibrous streak
28	Gonadoblastoma Sertoli granulosa cells surrounding round eosinophilic laminated areas Calcifications Seminiferous tubules at the periphery	Present
26	Streak gonads Gonadoblastoma present in one of them	
9	Gonadoblastoma Calcifications	Fibrous cortex Gonadoblastoma Calcifications
47	Dysgerminoma Gonadoblastoma Calcifications Call Exner bodies Fibrous stroma	The same features as in right ovary except that the stroma was more predominant
25	Fibrous tissue	Gonadoblastoma
2? case 3 JS	Gonadoblastoma Calcifications Interstitial cell hyperpla a Poorly developed seminiferous t bules	Immature dysgenetic gonad

DISCUSSION

From the 25 cases previously published (Tabl s 3 and 4) as well as the case published in this report some characteristic features emerge

Gonadoblastoma (gonocytoma III) was found in cases both of female and male legal sex The cause of admission in patients brought up as females is usually primary amenorrhea and/or some signs of masculin

ization In girls precocious puberty is the sign which should alert one to the possibility of gonadal tumour Only in three cases (15 16 41 47) the cause of admission was pain in the lower abdomen In all these last cases a tumour was palpable in abdomen preoperatively Only three cases brought up as males have been published previously (22 23 58) it seems that undevelopment or ambiguity of external genitalia is the cause of admission in these individuals

In all cases there was abnormal gonadal development Neoplastic changes were found in fibrous stroma or in undeveloped dysgenetic testes In cases in which one gonad was completely destroyed by neoplasia the contralateral gonad was found to be a fibrous streak or dysgenetic testis In two cases the bilateral tumours were found in the place normally occupied by the ovaries

The feminine appearance of patients despite the presence of the Y chromosome is evidence that the gonads in which the neoplastic process arise were not properly developed

In some cases other types of gonadal neoplasm most often seminoma (dysgerminoma) were found (2 38 41 47 55) The findings of the other types of neoplasm is not surprising in view of the histological similarity of the tumours and because the malformed dysgenetic gonads are a suitable site for neoplastic development

In many tumours Sertoli granulosa cells surrounding laminated eosinophilic bodies were found In some of them the central part was calcified In some patients these structures were also found in contralateral dysgenetic gonads with no evidence of malignant change According to some authors these structures represent intratubular eggs (6 8) but according to others displaced spermatogonia (3) Leaving apart this problem it is interesting to note that such structures were found quite often in ovotestes in cases of testicular dysgenesis with scrotal or cryptorchid testes testicular feminization syndrome and pure gonadal dysgenesis with male karyotype (3 8 14 55 60)

In all cases in which a more detailed description of tumours is given calcifications were found It is worth noting here however that only in a *few of them was the presence of calcifications helpful in preoperative* radiological diagnosis (40 53 57) This may be because no radiological examination was done or reported in some cases or because calcifications were too small for detection as it was in our case In most cases signs of virilization and high levels of 17 ketosteroids points to the hormonal activity of these tumours In all cases in which gonadotrophins were evaluated the titer was found to be elevated (2 13 40 52 54 55 56 57)

In most cases the tumour was discovered during exploratory laparotomy the reasons for which were concerned with gonadal dysgenesis though it must be noted that in many cases the signs of masculinization were or should lead to the suspicion of gonadal neoplasm

From data collected in Table 3 (and especially the case G K of

Melicow in which laparotomy was done more than once) it can be seen that the tumour is slowly growing and relatively benign which is in accordance with the histological pattern This shows more differentiation that in other types of germ cell tumours (gonocytomas) (48 49 50) however it should be noted that gonadoblastoma was often found in both gonads (4 5 9 15 16 33 40 47 55) Therefore in cases in which gonadoblastoma is found in one gonad the contralateral gonad should be examined histologically during operation or removed especially when it is completely dysgenetic or capable of hormonal production contrary to the legal sex or the sex chosen by the patient

The postoperative prognosis is good There have been only two cases of postoperative death reported They are the I C of *Scully* (41) and the first case of *Fraser et al* (15 16) It must be noted that in neither of these cases can death be directly attributed to the neoplasm In the first case in which no autopsy was performed was no clinical evidence of tumour recurrence and the patient was reported to have died of rheumatic heart disease and uremia The second case died of chronic renal failure due to chronic and subacute glomerulonephritis There was no evidence of recurrence of tumour at autopsy The third case which should be mentioned here was that of *Perrin & Landing* (33) in which at autopsy of a 14 year old girl with severe chronic glomerulonephritis and undiagnosed pituitary stalk oligodendroglioma bilateral gonadoblastoma were found The presence of uremia in the case of Scully and the glomerulonephritis in the last two cases can be simply a coincidence but it should also raise the question whether in these cases the renal failure has a common etiological background with gonadal dysgenesis

The result of cytogenetic analysis in the case presented is in accordance with data published previously (35) As it can be seen from Table 3 in all cases in which gonadoblastoma was diagnosed a Y chromosome was present In 9 cases normal male 46/XY karyotype (9 15 16 43 47 53 54 55 57 58) and in 4 cases XO/XY mosaicism were found (4 5 25 40 52 53) In one case XY/XX mosaicism (30) and in one case XO/X minute metacentric chromosome (26) were encountered In 10 cases in which cytogenetic analysis was not performed a negative sex chromatin pattern was found in all (2 13 22 23 28 39 41) except one (33) in which a low positive sex chromatin count was found at autopsy

In comparison between cases of seminoma (dysgerminoma) and gonadoblastoma two factors seem to be of interest

Seminoma (dysgerminoma) was found both in cases with gonadal abnormalities (10 11 22 23 24 29 34 36) and in apparently normal fertile women (31 32 51) Gonadoblastoma has only been found in cases of abnormal gonadal development

The histological pattern of seminoma (dysgerminoma) is more immature and it must be noted that death apparently caused by semin ma

(dysgerminoma) have been reported In gonadoblastoma (gonocytoma III) tumour recurrence has never been reported and complications arise either from abnormal hormonal production or from twisting of the tumour

The histological and cytogenetic findings (Tables 3 and 4) strongly suggest that gonadoblastoma arises in dysgenetic testicular tissue

1 Gonadoblastoma was found in dysgenetic testis or dysgenetic gonads which often contained testicular remnants

2 In all cases in which cytogenetic analysis was performed a Y chromosome was present most often in a normal male 46/XY karyotype

3 In several investigated cases of gonadoblastoma characteristic so called intratubular bodies were found appearing within tumoral nests in proximity of these nests or in contralateral not involved gonad Such formations have been found also in dysgenetic testes or in dysgenetic rudimentary gonads in patients with male karyotype (3 14 55)

SUMMARY

A boy with ambiguous external genitalia testicular dysgenesis with gonadoblastoma (gonocytoma III) and XO/XY karyotype is presented The clinical histological and cytogenetic findings in previously published cases with gonadoblastoma (gonocytoma III) are reviewed Gonadoblastoma is a relatively benign tumour found in malformed gonads in cases of abnormal sex determination and differentiation It arises from testicular remnants or dysgenetic testes In cases in which cytogenetic analysis was performed a Y chromosome was always present most often in a normal male 46/XY karyotype

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THE VASCULAR SUPPLY OF HEALING WOUND

An Angio and Histoangiographic Study

By

ARIKI O KARIPINEN and HANNU MYLLARNIEMI

Received 8 iv 67

Little study has been devoted to the vascularization of wounds although this obviously plays an important part in wound healing The knowledge to be gained from histological studies of vascular regeneration is scanty (see *Allgower* 1956) The investigations have shown that in the stage of fibroplasia newly formed blood vessels arise as sprouts of proliferating endothelial cells At first these sprouts are solid tendrils of cells but later they acquire a lumen and blood flows through them (*Willis* 1961)

Only a few studies on the vascularization of the healing wounds exists (*Clark & Clark* 1939 *Hughes & Dann* 1951 *Bellman et al* 1960 *Smahel & Charvat* 1963 and *Schoefl* 1963) In the main these have been confined to the healing of unsutured skin wounds *Weiber* (1959) studied the vascularization of sutured wounds by the application of an isotope technique

According to *Weiber's* findings published in 1954 vascularization of the healing wound progresses steadily during the first 5 days although as late as 13 days after incision the vasculature is still about 50 per cent more abundant than in intact tissue

Smahel & Charvat (1963) reported that unsutured skin wounds healed within 15 days after intervention By the end of this period there was observed a gradual reduction of the vascular network in the region of the defect In the scar itself fewer blood vessels were discernible than in the undamaged neighbourhood

In the present study wound healing in laparotomized rats was examined by means of an angiographic and histoangiographic method

This study has been supporte[illegible] a grant fr[illegible]m the *Pe[illegible] Os[illegible]ar Klinge[illegible]ahl Foun[illegible]a[illegible]tion* to which the authors ar[illegible] The auth[illegible] also wish to express the[illegible]r gratitude to Professor *C F L[illegible]* MD Docent [illegible] Radiology and the *S[illegible] Jusélius Foun[illegible]ation* [illegible] our dispos[illegible]l the roentgen equipment used in the inve[illegible] F[illegible]rthern[illegible]re the authors a[illegible]e indebted to Mis[illegible] *Ann Kristin Thors* and M[illegible] *R[illegible] Nurme* for their a[illegible]tanc[illegible] th[illegible] the h[illegible]tol[illegible]gical sections

MATERIAL

The material consisted of 38 male albino rats at mean weight of 150 g.

METHODS

Experimental: after ether anaesthetization a laparotomy wound of invariable length (5 cm) was produced under conditions which were as strictly aseptic as possible. The muscle and skin cut was sutured with continuous atraumatic chromic catgut. Angiography was performed after 3, 6 and 12 hours and after 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 days.

Angiographic: The angio- and histoangiographic method (*Karppinen & Myllärniemi* 1967) is only briefly outlined here.

In the method used the vascular bed of killed animals was filled with a mixture of roentgenological contrast medium (Micropaque, Damancy & Co. Ltd., England) and dye chinese ink (Pelikan Auszieh Tusche) which was injected into the cannulated descending aorta within physiological pressure of the experimental animal. Specimens for further examination were taken 4–6 hours later and were fixed in 10 per cent neutral formalin for 2–3 days. After fixation they were radiographed on Kodak Microtex X-ray film.

For histoangiographic and histological examination the specimens were treated in a rising alcohol series and xylol, embedded in paraffin (melting point 54° C) and sectioned at 5, 20 and 150 μ. The thin sections were stained with haematoxylin-eosin and *Van Gieson* and the thick sections were analysed unstained under a *Leitz* Microscope type Lanphot with mercury lamp.

RESULTS

The results have been compiled in Table 1.

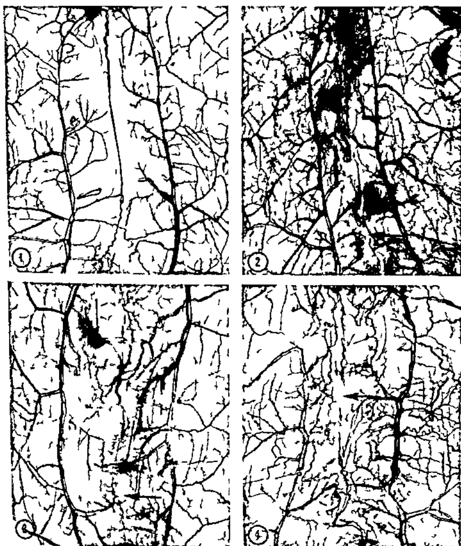
TABLE 1

Rough Estimate of Different Parameters in a Healing Laparotomy Wound in the Rat

Time after intervention	No. of animals	Vasodilatation wound	Vasodilatation periph.	Newly formed blood vessels	Inflammatory cells	Fibroblastic proliferation
3 hours	2	—	—	—	++	—
6 hours	4	++	+	±	+++	—
12 hours	3	+++	+++	±	+++	—
1 day	5	+++	+++	++	+++	—
2 days	5	+++	++	++	++	+
4 days	4	+++	+	++	+	++
6 days	4	++	—	+++	—	+++
8 days	3	+	—	++	—	+++
10 days	3	—	—	++	—	+++
12 days	2	—	—	+	—	++
14–18 days	3	—	—	+	—	+

— not observable
\+ slight or scanty (<10 parameters in scene)
++ moderate or relatively abundant (intermediate)
+++ marked or abundant (innumerable)
(the inflammatory cells around the sutures are not taken into account here)

Vasodilatation (dilated vessels seen in angiograms) was the first phenomenon observable after intervention. Its onset was *noted after* 6 hours and after 12 hours a large area of the abdominal wall was in



Figs 1–4

Fig 1 Angiogram × 2 from the intact abdominal wall of the rat The epigastric artery and vein are seen on both sides of the linea alba The cutis and sub cutis have been excised (as in all spe imens)

Fig 2 Angiogram × ? of a 24 hour old laparotom wound (thick arrow) Intense vasod latation is observable throughout the entire abdominal wall

Fig 3 Angiogram × 2 of a 6 day old laparot my wound (thick arrow) Peripheral vasodilatation has subsided Blood e els a e m st nume ous round about the sutures where an ext asati n of c nt st med um from newly formed vessels is seen (long arrows) N te th bl o i v el invasion over the sutures (short arrows)

Fig 4 Angiogram × 2 of an 18 da old l parotom wound (thick arrow) The vascular reaction h s ub ded except in th urround ngs of the sutures (short arrows) A vas al r bridge is een n the pe itone l surface of the abdominal wall (this i best seen in stereoangiograms)

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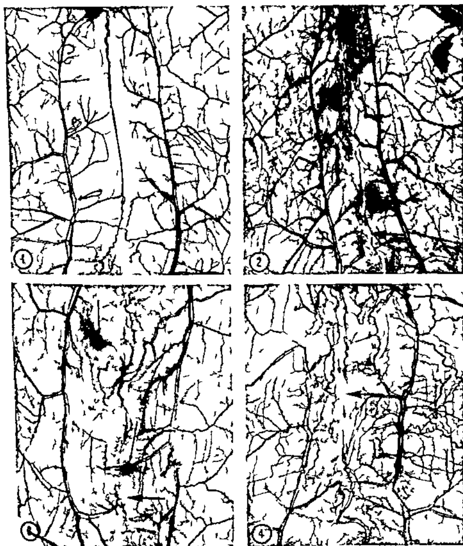
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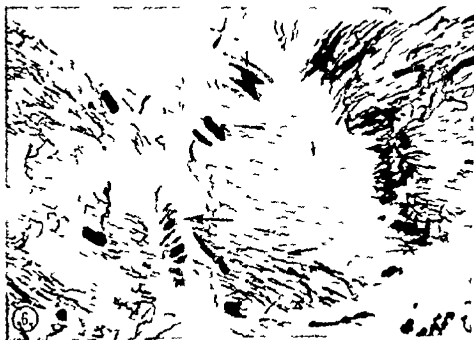
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Fig 4 Angiogram × 2 of an 18 day old laparotomy wound (thick arrow) The vascular reaction has subsided except in the surroundings of the sutures (short arrows) A vascular bridge is seen on the peritoneal surface of the abdominal wall (this is best seen in stereoangiograms)


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volved 6 days after intervention vasodilatation was no longer detectable in the abdominal wall but persisted in the wound area from which it gradually disappeared becoming completely absent by the 10th day Vasodilatation was clearly discernible both in the angiograms (Figs 2 and 3) and in the histoangiograms (Fig 5) and the findings mutually corresponded In some rats a local focus of infection was observable in the wound area which caused vasodilatation of longer duration than in uninfected cases However infected cases were omitted

The formation of blood vessels As early as *6 hours* after intervention some minute and poorly distinguished vessels were seen extending towards the wound (Fig 5) A close study of the process was practicable only after 24 hours (Figs 2 and 6) The density of blood vessels in the wound area was highest on the 6th day (Figs 3 and 7) It then gradually diminished so that after 10–12 days only a few blood vessels were seen these were mostly found in the neighbourhood of the suture and surrounding it (Figs 4 and 8) During the process of formation of new blood vessels the suture also appeared to play an important part in other respects If the wound was tightly sutured each suture was invariably surrounded by an avascular area (Fig 6 small arrow) which was in turn surrounded by a dense vascular network The vascular invasion across the wound also seemed in part to utilize the sutures crossing the wound This was more clearly observable on the peritoneal surface than elsewhere (Figs 4 and 8)

It was found that the blood supply of the healing wound derived from three sources the most important part being played by the vascular system of the abdominal muscles Furthermore the omentum and testicular fat fringe functioned as intra abdominal sources of vascular regeneration Occasionally it was observed that the wound was substantially supplied with capillaries from these structures Fig 5 illustrates that the subcutaneous blood vessels were the first to react after closure of the wound and it is obvious that this system also serves to supply the wound

Histological findings In the wound area and its neighbourhood the appearance of inflammatory cells was observed 3 hours after intervention and was found to reach its maximum after 6–24 hours Subse

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Figs 5–6

Fig 5 Histoangiogram × 2[illegible] of a 150 μ thick unstained section of a 6 hour old laparotomy wound (thick arrow) in the area of which the beginning of v[illegible] dilatation is seen Large vessels are to be seen on the peritoneal aspect of the wound (P) Note the subcutaneous dilated vessel (S) Suture is marked with short arro[illegible]

Fig 6 Histoangiogram × 26 of a 2 day old laparotomy wound (thick ar[illegible] Most of the [illegible]sels are seen surrounding the avascular area near the suture (short arrows)





Figs 7 8

Fig 7 Histoangi gram × 26 of a 6 day-ol l lapar t my wound (thick arr w) N t *the diffuse vascularization of the w und The area near the suture* (sl rt arrow) has been invaded by numerous vessel

Fig 8 Histoangiagram × 26 of a 10 day-old lapar t my wound (thick arr w) On the peritoneal aspect f the wound (P) bl o l vessels are still present r d about the chromic suture (short arrow)

quently the inflammatory cells persisted in the surroundings of the suture throughout the period of observation. Fibrocytes appeared in the wound area after *two days* and gradually increased in number. After 6–10 days fibroblastic proliferation attained its maximum.

DISCUSSION

The present study was principally aimed at examination of the vascularization of healing wounds which had been produced by laparotomy and sutured with chromic catgut. The first vascular reaction, vasodilatation in the wound area and in an extensive surrounding area of the abdominal wall was found to begin almost immediately, *i. e.* about 6 hours after intervention. Vascular regeneration was most intensive after 6 days and persisted for a lengthy period in the surroundings of the suture. The present findings are in close agreement with those obtained by previous investigators (see introduction). The observation that the suture plays an important role in vascularization of the wound has not been reported earlier. As a rule the most vigorous vascular reaction was found to occur round the suture. It is apparent that tension and the relative anoxia resulting from it are of some importance in this connection. Nevertheless throughout the period of the observation the most marked inflammatory reaction was observed in the neighbourhood of the suture. It appears that this process was conducive to an increase and prolongation of vascularization, just as was observed as regards wound infection.

The density of newly formed blood vessels in the wound area was highest after 6 days. The simultaneous occurrence of vasodilatation strengthened this impression. Blood vessels were usually more abundant in the close vicinity of the wound area than in the wound area itself, *i. e.* in the developing granulation tissue.

The method applied made it possible to study both the density of the vascular network and the vasodilatation, whereas an isotope technique for instance does not allow of separate estimation of these two phenomena. The latter technique yields information only of matters concerning the total volume of vessels in the wound, which *Weiber* (1959) reported to be at its maximum in the rabbit on the 5th day after intervention. It appears that the isotope technique is valuable in the study of wound vascularization but is inadequate as the sole method and must always be combined with some angiographic method.

The employment of histoangiography made visualization of new blood vessels easier than did angiography (compare figures 2 and 6 with each other). By virtue of vasodilatation numerous blood vessels which were indiscernible in the abdominal wall of the intact animal became visible after laparotomy. (Note the marked difference in Figs. 1 and 2). Later, after 2–4 days, when formation of new blood vessels was more abundant, their detection in the angiograms presented no

difficulties The most intensive vascular reaction was concurrent with the beginning of the strongest fibroblastic activity in the wound area but it needs emphasis that new blood vessels began to form at a point of time which was definitely earlier This argues in favour of the view that the regeneration of blood vessels at least in part and particularly in the initial stage of wound healing occurs irrespective of the fibroblastic activity in the wound

The experimental work by *Schoefl* (1963) concerning growing capillaries in unsutured cremaster muscle wound of the rat indicates that two weeks after intervention the wound area had become an inconspicuous vascular tangle After this time interval scars gradually diminished in size and became progressively more avascular This is not in agreement with our results The regenerative response in the vascular bed in the abdominal wall of the same animal was more rapid especially it started and diminished earlier This discrepancy may not be explained by the fact that the contrast medium did not penetrate all the blood vessels present in the wound area since the vascular bed round about the sutures was always well filled with contrast medium seen in both angio and histoangiograms The histological examination was also used for a check of the findings in angio and histoangiographs which according to *Rubin* (1964) is necessary One possible explanation is that the rapidity of the vascular regeneration is related to the location of the wound in the body *Butcher* (1964) has stated that the rapidity of wound healing differs in different parts of the body

After third degree burns some research workers have failed to observe any vascular reaction during the first week (*Order et al* 1965) It is apparent however that in the study concerned the capillary net work escaped attention in that the radiopaque mass employed (*Schlesinger* 1957) did not penetrate the capillaries In studies on the vascularization of granulation tissue accordingly this material might be omitted

SUMMARY

Vascularization of a healing laparotomy wound has been studied by angio- and histoangiography with the purpose to determining the factors of essential importance considered from the standpoint of vascular regeneration It was found that a vascular reaction occurred very soon after intervention and reached its maximum after 6 days It then subsided gradually and disappeared within 18 days In the initial stage in particular vascularization occurred independently of the fibroblastic activity in the wound The suture used to close the wound played an important part in vascularization The most vigorous reaction was usually observable round about the suture where it also persisted longest In addition an invasion of blood vessels across the wound to the other side occurred often over the sutures

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AN INQUIRY INTO THE TREPHOCYTIC FUNCTION OF THYMUS LYMPHOID CELLS IN LIVER REGENERATION

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There is an old theory that lymphocytes are of importance to cell growth, that they are trephocytes. This theory, combined with the fact that lymphocyte production in the thymus is intense (*Fichtelius* 1960, *Metcalf* 1964, 1966 (reviews)), gives rise to another theory, namely that one of the thymus functions is the export of lymphocytes or break-down products of lymphocytes which are used in the formation of new cells. This function has not been fully substantiated hitherto, although it is supported by circumstantial evidence of different kinds (*Fachet et al.* 1963, *Craddock et al.* 1964, *Dukor & Miller* 1965, *Fichtelius & Linna* 1967). A reutilization of lymphocyte DNA has been suggested and shown to occur, but it is also shown that lymphocytes are by no means the only DNA vectors (*Fichtelius & Linna* 1967 (review)). Most of the work on reutilization of DNA has been made with tritiated (^{3}H) thymidine.

It has been possible to show a quantitatively measurable migration of tritium labelled DNA from the thymus to other lymphoid organs by combining *intra thymus* labelling with ^{3}H thymidine with extraction of nucleic acids of different organs, DNA measurements and liquid scintillation counting of tritium (*Linna & Stillström* 1966, *Linna* 1967).

As this technique has been useful in the studies of thymus function in immunobiology, it could also have an application in the study of the other function suggested for thymus cells, namely the trephocyte function.

Partial hepatectomy is a forceful stimulus to hepatic regeneration. DNA synthesis increases from its normal low values to markedly elevated ones abruptly 15–18 hours after the operation. The incorporation of labelled precursors reaches a peak 22–26 hours after the opera-

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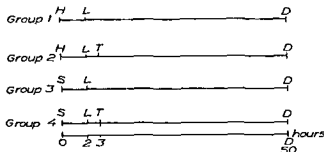


Fig 1
Experimental Schedule

H = partial hepatectomy
S = sham operation
L = local labelling of the thymus with ^{3}H thymidine
T = subtotal thymectomy
D = death

tion falls off somewhat and continues at elevated levels for some days (*Bucher* 1963 (review))

Thus by labelling partially hepatectomized animals *intra thymus* with ^{3}H thymidine and extracting DNA of liver and other organs it seemed to be possible to establish whether thymus cells could deliver labelled DNA and/or DNA degradation products to regenerating liver

MATERIAL AND METHODS

Animals

60 young male guinea pigs were taken from the same breeder and kept in quarters with darkness between 20 and 8 and light between 8 and 20 o clock for at least one week before the beginning of the experiment The animals weighing 190–250 g at the beginning of the experiment were matched by weight into the following groups (Fig 1)

Group 1 13 animals These animal were partially hepatectomized and labelled *intra thymus* with ^{3}H thymidine two hours later The animals were killed 50 hours after partial hepatectomy 48 hours after administration of label

Group 2 13 animals These animals were partially hepatectomized labelled *intra thymus* with ^{3}H thymidine two hours later and subtotally thymectomized still one hour later The animals were killed 50 hours after partial hepate tomy 48 hours after administration of label

Group 3 16 animals These animals were ham perated concern ng hepatectomy and labelled *intra thymus* with ^{3}H thymi line two hour l ter The animals were killed 50 hours after sham operation 48 h ur fter adm nistration of label

Group 4 16 animal These animal w r h n p ated on ern ng hepatectomy labelled *intra thymus* with ^{3}H th m lin tw h l t r and s ıbt tally th mec tomized still one hour later The n mal were killed 0 l u after sham operation 48 hours after administration of l l el

Group 5 2 animals These an m l l e ng w thin th w ght rang given above were not gi en isotope an l w lat r u c l f r ba kg nd orrections n tr tium measurements (see below)

Operations

a Partial hepat ctomy The partial hepatectomies were made at the same t me f the day 8–10 o clo k be au e f the cir ad an rhythm of g ow th of the li The animal we e ane the i ed th an intrape itoneal inje t on of Nembut l®

(Abbot Lab Ltd Queenborough Kent England) The left lateral lobe and the left medial lobe were easily delivered securely ligated by silk and then excised The excised parts consisted of about 40 per cent of the total liver weight

b Sham operation The sham operations were made between 8 and 10 o clock a m The animals were anesthesized with Nembutal® as described above A similar midline incision was made and the liver lobes in question mobilized but not excised

c Intra thymus labelling The *intra thymus* injections were made according to a technique used previously (*Linna & Stillstrom* 1966) When necessary ether was used to prolong the nembutal anesthesia Each animal was given 20 µC of ^{3}H thymidine (spec act 6 7 C/mM 0 036 mg thymidine/ml New England Nuclear Corp Boston Mass USA) diluted to a volume of 50 µl with physiological saline Half the dose was placed in each thymus lobe Thus the animals were given 0 08 0 1 µC of ^{3}H thymidine per g body weight *i e* 0 003–0 004 µg thymidine per g body weight The injections were made with a 100 µl Hamilton® syringe (Hamilton Comp Inc Whittier Calif U S A) with a gauge 22 needle (external diam 0 30 mm) The skin was closed with a couple of sutures

d Subtotal thymectomy The subtotal thymectomy was made according to *Gyllensten* (1953) in ether anesthesia By this method at least 95 per cent of thymus tissue can be quickly removed

Dissection Procedure

The animals were killed with ether and the thymus about 1 g of the liver the spleen the mesenteric lymph nodes bone marrow from both femurs about 0 5 g of duodenum and one testis were dissected out The duodenum samples were cut up and cleaned in 0 9 per cent saline The samples were divided into two of about the same weight and these were then treated as different samples All samples were put down in 5 ml of cold 5 per cent trichloroacetic acid (TCA) and then immediately frozen and stored at −20 C until extraction of nucleic acids was performed

A small piece of the liver was taken for histologic and autoradiographic study (see below)

Extraction of Nucleic Acids DNA Measurement and Liquid Scintillation Technique

The nucleic acids were extracted by a modified *Schneider* procedure (*Schneider* 1945) 5 per cent cold TCA was used The lipid and phosphoprotein removing steps were omitted The nucleic acids were extracted with 5 ml of 5 per cent TCA in a water bath at +90 C for 30 minutes The DNA content of the supernatant was measured with *Burton s* modification (*Burton* 1956) of the diphenylamine reaction

The radioactivity was measured in a liquid scintillation counting system (Packard Tri Carb 314 ex) at +2 C using 10 ml of *Bray s* solution as scintillator (*Bray* 1960) to which 1 ml water phase (nucleic acids in 5 per cent TCA) was added Nucleic acids isolated in the same way from the corresponding organ of the non injected animals were used for background correction The channels ratio method (*Baillie* 1960 *Herberg* 1965) was applied to control quenching Each sample was counted twice and the mean after background subtraction of these two values was used in the calculations of specific activity (spec act) More detailed descriptions of these methods are to be found elsewhere (*Linna & Stillström* 1966 *Linna* 1967)

Histologic and Autoradiographic Technique

The liver pieces destined for autoradiographs were placed in 4 per cent carbonate buffered formalin for subsequent ethanol dehydration and xylene clearing according to the usual methods before being embedded in paraffin wax 5 µ sections were cut The autoradiographic technique was largely that described by *Pelc* (1956) The slides were exposed at about +4 C for two months The sections were stained with haemalum according to Mayer A more detailed description of the autoradiographic method used is to be found elsewhere (*Fichtelius & Linna* 1965)

In the examinations of the slides 20 view fields containing about 170 cells each were scanned in each slide with microscope magnification 540 x Where presumably labelled cells were found they were studied in detail with magnification 1000 x *Cells containing* more than 5 grains were registered concerning cell type and number of grains overlying the nucleus

Calculations

(The facts and assumptions leading to the use of these symbols and calculations, are explained in Discussion pages 5-10

Symbols

spec act = specific activity expressed as counts/min/mg DNA
rel act = relative activity = spec act of an organ divided by the sum of spec act of all the organs sampled except the thymus of the same animal (i e liver spleen mesentric lymph nodes bone marrow duodenum and testis)
M_T = mean of spec act of an organ animals with thymus left *in situ*
M_C = mean of spec act of an organ in thymectomized animals
$M_{Q(T)}$ = mean of rel act of animals with thymus *in situ*
$M_{Q(C)}$ = mean of rel act of thymectomized animals
$\varepsilon_{M_{Q(T)}}$ = standard error of $M_{Q(T)}$
$\varepsilon_{M_{Q(C)}}$ = standard error of $M_{Q(C)}$

The following calculations were performed

1 The DNA values for duplicate samples were added together and so were the values of counts/min and these figures were used for the calculations of spec act *On some occasions one of the duplicate sample values was missing and in these cases only the remaining duplicate sample gave the spec act*

2 The spec act and rel act of each organ M_T M_C $M_{Q(T)}$ $M_{Q(C)}$ $\varepsilon_{M_{Q(T)}}$ and $\varepsilon_{M_{Q(C)}}$ were calculated for all organ samples

3 Student s t test was applied to compare the relative activities by the formula

$$t = \frac{M_{Q(T)} - M_{Q(C)}}{\sqrt{\varepsilon_{M_{Q(C)}} + \varepsilon_{M_{Q(T)}}}}$$

RESULTS

Groups 1 and 2 In these partially hepatectomized animals the mean of spec act was of largely the same magnitude for all the organs examined in the group of animals with the thymus left *in situ* as in the thymectomized ones (Table 1)

TABLE 1

Partially Hepatectomized Animals Mean of Spec Act of Different Organs of Animals with Thymus in situ *(M_T) and of Different Organs of Thymectomized Animal (M_C)*

Organ	Thymus	Liver	Spleen	M lymph nodes	Bone marrow	Testis	Duod
M_T	4 705	10	251	241	503	256	771
M_C	4 764	14	292	194	451	253	682

The spec act are of largely the same magnitude for each organ when groups 1 (M_T) and 2 (M_C) are compared

(Abbot Lab Ltd Queenborough Kent England) The left lateral lobe and the left medial lobe were easily delivered securely ligated by silk and then excised The excised parts consisted of about 40 per cent of the total liver weight

b Sham operation The sham operations were made between 8 and 10 o clock a m The animals were anesthesized with Nembutal® as described above A similar midline incision was made and the liver lobes in question mobilized but not excised

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In the examinations of the slides 20 view fields containing about 170 cells each were scanned in each slide with microscope magnification 540 x Where presumably labelled cells were found they were studied in detail with magnification 1000 x Cells containing more than 5 grains were registered concerning cell type and number of grains overlying the nucleus

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(The facts and assumptions leading to the use of these symbols and calculations are explained in Discussion pages 5 10

Symbols

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rel act = relative activity = spec act of an organ divided by the sum of spec act of all the organs sampled except the thymus of the same animal (i e liver spleen mesentric lymph nodes bone marrow duodenum and testis)
M_T = mean of spec act of an organ animals with thymus left *in situ*
M_L = mean of spec act of an organ in thymectomized animals
$M_{Q(T)}$ = mean of rel act of animals with thymus *in situ*
$M_{Q(C)}$ = mean of rel act of thymectomized animals
$^{\varepsilon}M_{Q(T)}$ = standard error of $M_{Q(T)}$
$^{\varepsilon}M_{Q(C)}$ = standard error of $M_{Q(C)}$

The following calculations were performed

1 The DNA values for duplicate samples were added together and so were the values of counts/min and these figures were used for the calculations of spec act On some occasions one of the duplicate sample values was missing and in these cases only the remaining duplicate sample gave the spec act

2 The spec act and rel act. of each organ M_T M_C $M_{Q(T)}$ $M_{Q(C)}$ $M_{Q(T)}$ and $^{\varepsilon}M_{Q(C)}$ were calculated for all organ samples

3 Student s t test was applied to compare the relative activities by the formula

$$t = \frac{M_{Q(T)} - M_{Q(C)}}{\sqrt{^{\varepsilon} M_{Q(C)} + ^{\varepsilon} M_{Q(T)}}}$$

RESULTS

Groups 1 and 2 In these partially hepatectomized animals the mean of spec act was of largely the same magnitude for all the organs examined in the group of animals with the thymus left *in situ* as in the thymectomized ones (Table 1)

TABLE 1

Partially Hepatectomized Animals Mean of Spec Act of Different Organs of Animals with Thymus in situ (M_T) and of Different Organs of Thymectomized Animals (M_C)

Organ	Thymus	Liver	Spleen	Mes lymph nodes	Bone marrow	Testis	Duod
M_T	4 705	130	251	241	503	256	771
M_C	4 764	146	222	194	451	53	632

The spec act are of largely the same magnitude for each organ, when groups 1 (M_T) and 2 (M_C) are compared

The mean of rel act of the animals with their thymus *in situ* was higher than mean of rel act of the thymectomized ones for liver samples (0 10 vs 0 08) but the differences were not significant The rel act values of all the other organs examined were rather close to each other (Table 3)

The screening of autoradiograms revealed low background Very few cells contained more than 1–2 grains Only very few cells containing more than 5 grains were found in the whole series of slides The screened part of each slide never contained more than a single cell with more than 5 grains No cells with more than 12 grains were found The labelled cells were hepatic parenchymal cells and mesenchymal cells No lymphocytes containing more than 5 grains were found The labelled cells were no more frequent in the group of animals with their thymus left *in situ* than in the thymectomized ones

TABLE 2

Sham—Operated Animals Mean of Spec Act of Different Organs of Animals with Thymus in situ (M_T) and of Different Organs of Thymectomized Animals (M_C)

Organ	Thymus	Liver	Spleen	Mes lymph nodes	Bone marrow	Testis	Duod
M_T	10 072	303	538	599	1 263	544	1 988
M_C	9 496	553	675	660	1 907	691	2 467

The spec act are of largely the same magnitude for each organ when groups 3 (M_T) and 4 (M_C) are compared

Groups 3 and 4 In these sham operated animals the mean of spec act was of largely the same magnitude for all the organs examined in the group of animals with the thymus left *in situ* as in the thymectomized ones (Table 2)

The mean of the rel act of the animals with their thymus *in situ* was higher than the mean of rel act of the thymectomized ones for liver samples (0 13 vs 0 08) but the differences were not significant The rel act values were rather close to each other for all the other organs examined (Table 4)

Also in these autoradiograms the background was low Very few cells contained more than 1 2 grains Only single cells containing 5–12 grains were found These cells were hepatic parenchymal cells and mesenchymal cells There were no clear differences between groups 3 and 4 nor between the partially hepatectomized and sham operated animal

DISCUSSION

The reutilization of DNA degradation products of dying lymphoid cells is well known also in hepatic regeneration (*Bryant* 1962 *Robin*

TABLE 3

Partially Hepatectomized Animals Mean and Standard Error of the Mean of Rel Act of Different Organs of Animals with Thymus in situ ($M_{Q(T)} \pm \varepsilon_{M_{Q(T)}}$) and the same Figures of Thymectomized Animals ($M_{Q(C)} \pm \varepsilon_{M_{Q(C)}}$)

Organ	Thymus	Liver	Spleen	Me lymph nodes	Bone marrow	Testis	Duod
$M_{Q(T)} \pm \varepsilon_{M_{Q(T)}}$	16±0 30	0 10±0 01	0 11±0 01	0 11±0 00	0 22±0 02	0 11±0 01	0 35±0 02
$M_{Q(C)} \pm \varepsilon_{M_{Q(C)}}$	2 48±0 3	0 08±0 01	0 11±0 01	0 10±0 01	0 22±0 01	0 11±0 01	0 35±0 01

Q(T) = rel act = spec act of an organ divided by the sum of spec act of all the organ sampled except the thymus of the same animal with thymus *in situ*

Q(C) = rel act = spec act of an organ divided by the sum of spec act of all the organs sampled except the thymus of the same thymectomized animal

$M_{Q(T)}$ = mean of Q(T)

$M_{Q(C)}$ = mean of Q(C)

$\varepsilon_{M_{Q(T)}}$ = standard error of $M_{Q(T)}$

$\varepsilon_{M_{Q(C)}}$ = standard error of $M_{Q(C)}$

The $M_{Q(T)}$ values of liver are higher than the $M_{Q(C)}$ of this organ but the differences are not significant The other $M_{Q(T)}$ values are rather close to the $M_{Q(C)}$ values of the same organ

TABLE 4

Sham Operated Animals. Mean and Standard Error of the Mean of Rel. Act. of Different Organs of Animals with Thymus in situ $(M_{Q(T)} \pm \varepsilon M_{Q(T)})$ *and the same Figures of Thymectomized Animals* $(M_{Q(C)} \pm \varepsilon M_{Q(C)})$

Organ	Thymus	Liver	Spleen	Mes. lymph nodes	Bone marrow	Testis	Duod.
$M_{Q(T)} \pm \varepsilon M_{Q(T)}$	2.01 ± 0.20	0.13 ± 0.03	0.10 ± 0.00	0.11 ± 0.01	0.25 ± 0.02	0.11 ± 0.01	0.36 ± 0.02
$M_{Q(C)} \pm \varepsilon M_{Q(C)}$	1.77 ± 0.14	0.08 ± 0.01	0.10 ± 0.00	0.11 ± 0.00	0.21 ± 0.01	0.12 ± 0.01	0.38 ± 0.01

For definitions of Q(T), Q(C), $M_{Q(T)}$, $M_{Q(C)}$, $\varepsilon M_{Q(T)}$ and $\varepsilon M_{Q(C)}$ see legend to Table 3.

The $M_{Q(T)}$ value of liver are higher than the $M_{Q(C)}$ values of this organ, but the differences are not significant. The other $M_{Q(T)}$ values are close to the $M_{Q(C)}$ values of the same organ.

son & Brecher 1963) Because of the risks of cell damage of purely traumatic nature at the *intra thymus* injections the volumes of isotope used for labelling were kept small The dose of isotope was kept as small as possible to avoid radiation damage (*Wimber* 1964 *Thrasher* 1966 (reviews)) The administration of label directly into a fast proliferating lymphatic organ motivates still more the use of a low dosage although a very small part of the administered label will stay within the thymus Another reason for keeping the dose of thymidine low is the possible mitosis stimulating effect of thymidine (*Greulich et al* 1961) The dose used in this study (max 0 004 μg per g body weight) is much lower than the threshold value given by *Greulich* (1962) for the mitosis stimulating effect Also chromosome aberrations in tissue cultures have been reported after addition of large amounts of thymidine (*Yang et al* 1966)

A 40 per cent hepatectomy gives a good stimulus to hepatic regeneration with DNA formation (*Bucher & Swaffield* 1962 *Bucher* 1963) The normal fast break down of thymidine in the liver (*Nygaard & Potter* 1959 *Gerber & Remy Defraigne* 1963) can hardly be influenced as the dose of thymidine injected is so small and most of the hepatic tissue is present The normal availability time of ^{3}H thymidine of maximally one hour (*Cronkite et al* 1959 *Thrasher* 1966 *Chang & Looney* 1966) can not be expected to be prolonged and no labelled thymidine should be expected to leave the thymus after this time period

It is difficult for technical reasons to give all the animals exactly the same dose of isotope locally in exactly the same way especially as the quantities of ^{3}H thymidine must be kept rather small Because of that the spec act of all the samples show rather great interindividual variation Calculation of rel act i e division of the spec act of an organ by the sum of spec act of all organs examined except the thymus gives the advantage that variation of the values because of minor differences of isotope dose and administration is avoided as the labelling of each organ is related to the labelling of the other organ samples of the same animal The possibility of a transport of label of about the same extent to all the organs examined should not be revealed by the use of rel act but as the values of spec act are of largely the same magnitude in both the animals with the thymus left *in situ* and the thymectomized ones such a transport can hardly have been on a large scale The possibility of migration to other organs than the ones examined here is beyond the r[illegible]h of this study

The animals of groups [illegible] and 4 were thymectomized one hour after the *intra thymus* injection of ^{3}H thymi[illegible] It is highly probable that for every *intra thymus* in[illegible]tion there [illegible] curred a certain amount of lakage into the gene[illegible] reulation [illegible] magnitude of this leakage probably differs from [illegible] animal to [illegible] Thus all the organs (except the thymus) of [illegible] ups 2 and 4 [illegible] be considered to be labelled

TABLE 4

Sham Operated Animals Mean and Standard Error of the Mean of Rel Act of Different Organs of Animals with Thymus in situ ($M_{Q(T)} \pm \varepsilon M_{Q(T)}$) and the same Figures of Thymectomized Animals ($M_{Q(C)} \pm \varepsilon M_{Q(C)}$)

Organ	Thymus	Liver	Spleen	Mes lymph nodes	Bone marrow	Testis	Duod
$M_{Q(T)} \pm \varepsilon M_{Q(T)}$	2 01±0 20	0 13±0 03	0 10±0 00	0 11±0 01	0 25±0 02	0 11±0 01	0 36±0 02
$M_{Q(C)} \pm \varepsilon M_{Q(C)}$	1 77±0 14	0 08±0 01	0 10±0 00	0 11±0 00	0 21±0 01	0 12±0 01	0 38±0 01

For definitions of Q(T) Q(C) $M_{Q(T)}$ $M_{Q(C)}$ $\varepsilon M_{Q(T)}$ and $\varepsilon M_{Q(C)}$ see legend to Table 3

The $M_{Q(T)}$ values of liver are higher than the $M_{Q(C)}$ values of this organ but the differences are not significant The other $M_{Q(T)}$ values are close to the $M_{Q(C)}$ values of the same organ

sham operated animals of this experiment and the earlier migration study in the guinea pig One main difference is the way of obtaining information about leakage The combination of *intra thymus* labelling and thymectomy of the present experiment should give a true picture of the leakage labelling a short time after the administration of label and thymectomy but the thymectomy could influence the distribution of labelled lymphoid cells after 48 hours as the thymus humoral factor should not be synthesized and might not be available in normal amounts for this time after the operation

Also the sham operated animals had been subject to considerably more stress than required by the labelling procedure alone by anesthesia and the surgical procedure extending into the peritoneal cavity Thus many factors can have influenced the normal migration of cells or of labelled DNA from the thymus to the spleen

SUMMARY

Four groups of young male guinea pigs were used in this investigation Two of the groups were subjected to 40 per cent hepatectomy and given 20 μC of ^{3}H thymidine *intra thymus* two hours after hepatectomy The animals of one of these two groups were thymectomized one hour after labelling The two remaining groups were subjected to a sham operation concerning hepatectomy given 20 μC of ^{3}H thymidine *intra thymus* two hours later and the animals of one of these two groups were thymectomized one hour after labelling 48 hours after labelling the animals were killed and samples of liver and other organs were dissected out After isolation of nucleic acids DNA content and tritium activity were measured and spec act of the samples calculated By comparing the distribution of label in the animals with their thymus *in situ* and the thymectomized ones it was not possible to demonstrate a reutilization of thymus DNA label at hepatic regeneration with the experimental model used

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STUDIES ON THE COMPLEMENT FIXATION TEST WITH *MYCOPLASMA PNEUMONIAE* ANTIGEN

1 *Production of Antigen*

By

JAN ENG

Received 13 xii 66

In 1942–1945 *Eaton et al* (11 12 13 14) demonstrated that primary atypical pneumonia (pap) is caused by a micro organism as they found that sputum and lung tissue from patients with pap caused pulmonary infiltrates in cotton rats The effect could be neutralized by serum from pap patients in the convalescent phase Eaton et al also adapted and propagated the microorganism in chick embryos The first serological investigations on pap with the causal micro organism as antigen were carried out by *Eaton et al* (14 15) as neutralization tests in cotton rats and hamsters

In 1943 *Peterson et al* (35) and *Turner* (42) demonstrated that patients with pap develop raised serum titres of agglutinins against human erythrocytes on incubation at +4 C In 1945 *Thomas et al* (41) discovered that patients with pap develop raised serum titres of agglutinins against *Streptococcus MG*

In 1957 *Liu* (31) demonstrated Eaton agent directly in the respiratory tract of infected chick embryos using the fluorescent antibody technique In 1959 *Liu et al* (32) used the indirect fluorescent antibody technique with sections of infected chick embryos as antigen in titration of sera from patients with pap

The demonstration in 1961 by *Marmion & Goodburn* (34) of the sensitivity of Eaton agent to an organic gold salt strengthened the already existing hypothesis that Eaton agent belongs to the PPLO group of micro organisms (Pleuropneumonia like organisms or *Mycoplasmataceae* (17)) In 1962 *Chanock et al* (5) cultivated Eaton agent on an artificial medium and showed definitely that it belongs to the *Mycoplasmataceae* *Chanock et al* further isolated the organism directly from patients with pap on an artificial medium (7) and produced complement fixing antigen by propagation in a broth medium (6) In 1963 the organism was given the name *Mycoplasma pneumoniae* (4) There are now numerous investigations on the role of *M pneumo*

application of Dienes stain to the plate all the elements of growth showed strong affinity for the stain 3) The haemolysis test showed β haemolysis round all the elements of growth after 1–2 days

The broth culture was incubated aerobically at 37° C and shaken by hand once a day Growth became manifest by the formation of numerous floating colonies just visible macroscopically, that sank to the bottom leaving the supernatant medium relatively clear On quite slight movements the colonies whirled up causing clouding of the medium No subcultures were made in broth After incubation for 13 days the culture was filtered through several layers of sterile gauze to remove the agar cooled to +4 C and then centrifuged at 20 000 G for 45 min in a Sorvall superspeed RC 2B cooling centrifuge The deposit was washed once and then resuspended in 15 ml sterile saline giving 1/133 of the original broth volume tested for sterility by inoculation on blood agar and dextrose broth preserved by addition of Thiomersalat (Merthiolat) 1 10 000 (29) and stored at +4 C

Treatment of Anti Complementary Effect of the Antigen

Untreated suspensions of *M pneumoniae* are regularly strongly anti complementary We have previously tried to reduce the anti complementary effect of *M pneumoniae* suspensions by phenolizing the broth culture using the technique described by *Chanock et al* (6) but this method has not given satisfactory results in our hands However we found that extraction of the antigen with ether followed by precipitation in saline during evaporation of the ether under reduced pressure could reduce the anti complementary effect of the suspensions so that they could be used as CF antigens though in some cases the specific titre was also reduced Much the best method in our hands has been boiling in a water bath for 35 min and the antigen prepared from the Bård strain was therefore treated in this way Table 2 shows that the anti complementary titre thus sank from 1 32 to < 1 4 while the specific titre tested in the same experiment rose from 1 128 to 1 512 In these titrations serum from a rabbit immunized with the Bård strain

TABLE 2

The Effect of Boiling in a Water Bath for 35 min on the Anti Complementary Effect (Lower Rows) and on the Specific Complement Fixation Titre against Homologous Rabbit Immune Serum (Upper Rows) of Antigen from M pneumoniae Bård Strain The Figures in the Columns Show the Degree of Inhibition of Haemolysis

	Dilution of antigen								
	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024
Raw antigen	4	4	4	4	4	4	2	1	0
	4	4	4	3	1	0	0	0	0
Boiled antigen	4	4	4	4	4	4	4	3	1
	1	0	0	0	0	0	0	0	0

TABLE 3

Chessboard Complement Fixation Titration of Antigen Prepared from M pneumoniae Bård Strain against Homologous Rabbit Immune Serum (unbroken line) and against a Patient Convalescent Serum (Stippled Line) The Lines Show the Endpoint of Positive Reaction

Dil of serum	Dilution of antigen							
	1 4	1 8	1/16	1/32	1/64	1/128	1/256	1/512
1 5								
1 10								
1 20								
1 40								
1 80								
1 160								
1 320								
1 640								

propagated on rabbit PPLO broth was used as positive serum Table 3 shows a chessboard CFT titration of the boiled antigen against the rabbit immune serum and also against a convalescent serum from a patient with pneumonia showing significant rise of titre in the *M pneumoniae* CFT The boiled antigen was used in a 1 40 dilution for examination of patient sera *i e* the 2 litres of broth culture resulted in 600 ml antigen diluted for use

Production of Immunizing Antigen

The Bård strain was transferred from normal PPLO agar to rabbit PPLO agar and then passed times on that medium with 4–2 days intervals 300 ml rabbit PPLO broth was inoculated from the 4th and 5th rabbit agar passages Growth became manifest in the formation of numerous floating colonies which were just visible macroscopically After 14 days incubation the culture was filtered through gauze and centrifuged at 20 000 G for 45 min The sediment was resuspended in 6 ml saline checked for sterility and preserved with Merthiolate 1 10 000

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TABLE 2

The Effect of Boiling in a Water Bath for 3 min on the Anti Complementary Effect (Lower Rows) and on the Specific Complement Fixation Titre against Homologous Rabbit Immune Serum (Upper Rows) of Antigen from M pneumoniae Bird Strain The Figures in the Columns Show the Degree of Inhibition of Haemolysis

	Dilution of antigen								
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Raw antigen	4	4	4	4	4	4	2	1	0
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but PPLO broth but unlike these authors we also made up a rabbit PPLO agar medium for the adaption and preliminary passages of the strain before inoculation of the rabbit PPLO broth These subcultures on rabbit PPLO agar were carried out rapidly at intervals of 4–2 days —It is also hypothetically conceivable that individual rabbit sera may vary in their ability to support the growth of *M pneumoniae* An interesting finding in this connection is *Kenny & Grayston s* (27) demonstration of antibodies to *M pneumoniae* in normal rabbits *Klieneberger Nobel* (29) found that normal rabbit sera gave positive CF reactions with PPLO antigens in general and interpreted them as unspecific reactions

SUMMARY

Isolation of *M pneumoniae* and production of CF antigen mainly by the method of *Chanock et al* (6) is described The yield from 2 liters of broth culture was 600 ml antigen diluted for use

Very rapid growth on solid medium was obtained of the strain used for antigen production in some passages growth could be seen already after 24 hours Agar culture with rapid and heavy growth was used for inoculation of the broth for antigen production

Sera from different horses showed considerable variations in their ability to support the growth of *M pneumoniae* and serum from a horse giving good growth was chosen for preparation of media for antigen production

The marked anti complementary effect of untreated *M pneumoniae* suspensions was most effectively reduced by boiling in a water bath for 35 min with full retention or increase of the specific titre

Cultivation of *M pneumoniae* on rabbit meat broth enriched with rabbit serum for production of immunizing antigen in the preparation of specific rabbit immune serum is also described

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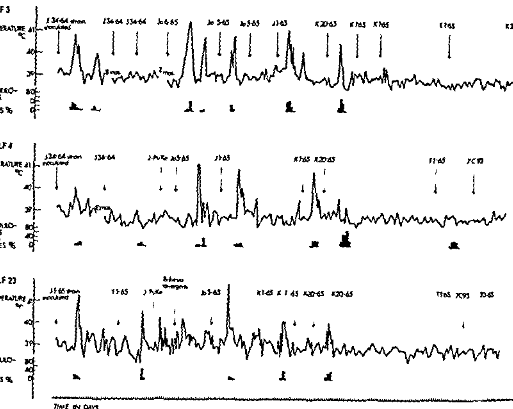


Fig 1

Temperature reactions and occurrence of tick borne fever bodies in four calves and two sheep inoculated serially with several heterologous strains of the causative agent (Remarks) Calf 3 had been splenectomized prior to the experiment calf 4 and sheep 2 were splenectomized during the experiments respectively 1 and 2¾ months before the homologous challenge with J34-64 strain

strain On occasion the interval was longer although a few inoculations were made at a time when the preceding reaction was still in progress As was stated in the first paper of this series in none of the 30 cattle or sheep in which homologous immunity against different strains was challenged within 3 months were any signs of tick borne fever reaction observed (9) Some of these challenge inoculations were made during the course of the serial inoculations to be described in this report In view of the above mentioned consistent finding of the development of homologous immunity and its apparent duration for at least a few weeks there was omitted actual demonstration of its presence by regular challenge prior to inoculation of each subsequent heterologous strain This had the advantage that a series of inoculations with several heterologous strains could be effected during a shorter period

In most of the serial inoculations the blood used had been stored in a frozen state (9)

RESULTS

Fig 1 presents the complete histories of serial inoculation with several heterologous strains in respect of 4 bovines and 2 sheep Table 1 is a simplified presentation of tests run with the remainder of the experimental animals 18 bovines and 12 sheep This indicates no more than

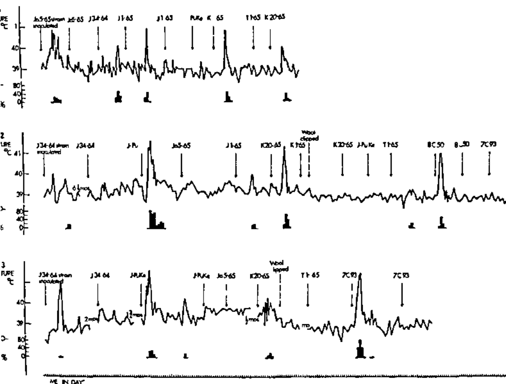


Fig 1 (cont)

whether the animals reacted detectably or did not react to each inocul ation made

These results indicate that it was rather exceptional for infection by any single strain to result in protection against a heterologous strain and that even after the experience of infection by several heterologous strains an animal could still react when again inoculated with a new strain In calf 4 reactions were recorded for 7 heterologous strains in calf 3 and sheep 2 for 6 strains and in calves 23 and 26 and in sheep 3 for 5 strains In all these animals the tests were conducted within a period of 6 months (in some of them during a considerably shorter period) counted from the subsidence of the first reaction or from the time of the last challenge by its ca is th strain to the inoculation of the last strain which still produced a det able reaction

The charts for calves 3 and 4 n t illustrate that a gradual development of broader cro n muni ned to take place during the course of increasing ex ence f a trains The last reactions in these calves were de b e on a t examination of blood smears no temperatur vas ol se t was surprising to find a reaction such as that c a by K20 a in calf 4 in which tick borne fever bodies we mingly a dant as those in a fully

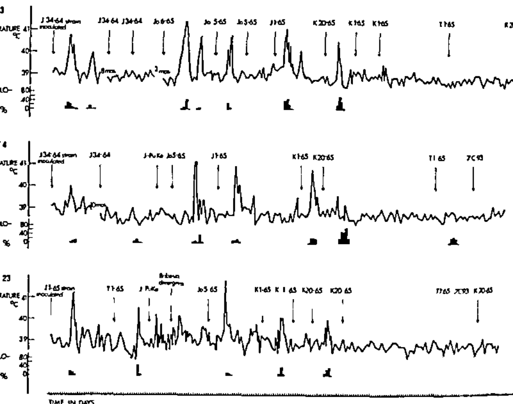


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Borne Fever Agent in 18 Cattle and 12 Sheep

of inoculations						Test animal
34 64)						Calf 1
34 64)	7 mos	(J34-64)	3 w	(J-Puke)	1 w	Calf 2
						Cow 7
-Puke)	2 w	Jo5 65				Heifer 13
-Puke)	1 w	(J-Puke)	10 d	Jo5 65		Heifer 14
						Cow 17
						Heifer 18
						Heifer 20
34 64)	2 w	Jo5-65	1 w	(J Puke)	1 w	Cow 21
Jo5-65)	1 w	(J34-64)	2 w	(J1-65)	2 w	Cow 22
1 65	3 w	K1 65	10 d	K20 65		Heifer 24
						Cow 28
						Cow 29
						Cow 32
						Heifer 33
						Heifer 36
						Heifer 37
						Calf 40
uke)	11 d	(Jo5-65)				Sheep 1
65	3 w	(K20-65)	1½ mos	8 C50		Sheep 4
						Sheep 5
						Sheep 6
Jo5-65) C50	2 w	(J1-6)	1 w	(J-Puke)	1 w	Sheep 7
20-65)	2 mos	8 C50				Sheep 8
1-65	3 d	(K20-65)	2 mos	(K20 65)	1 mo	Sheep 9
34 64)	2 w	K20 65	2 w	J Puke	3 w	Sheep 12
K20-65)	3 w	7 C93				Sheep 14
-65	1¾ mos	7 C93				Sheep 15
1 65)	3 w	(J-Puke)	1 w	(J-Puke)	11 d	Sheep 16
-65	0 d	J-Puke	5 d	(J-Puke)	2 w	Sheep 17

tectable reaction or did not along with the intervals between them
ose within parentheses indicate negative inoculations The intervals are measured either
action from the preceding inoculation to the next inoculation

new Finnish strains after experiencing fewer strains than did the animals included in Fig 1 Table 1 indicates that cows 21 and 22 evidenced a detectable reaction to J Puke and Jo5 65 strains alone these are the cases in which cross protection against other tested heterologous strains resulted from the least experience of different strains Standard techniques were applied throughout the present study and no examination was made of whether the immunity in cows 21 and 22 could have been broken down by massive doses of the heterologous strains tested

Apparently some strains were immunologically more closely related than others Tables 2 and 3 contain a summary of the results of all the

in Tables 2 and 3 The figures in parentheses indicate that this kind of lack of protection was encountered very frequently

If Fig 1 and Table 1 are further examined it is discovered that infection by J-Puke and K20-65 strains was never fully inhibited in sheep by virtue of previous experience of any combination of heterologous strains Although partial suppression was occasionally indicated as having taken place nevertheless every reaction by these strains was easily detectable In cattle all the inoculations by Jo5-65 strain produced a marked tick borne fever reaction The impression was gained that even the experience of several mainland strains (see Fig 1(9) for location of their origin) exercised no more than a slight effect if any on the course of the infection by this strain which originated from Aland archipelago On the contrary all the mainland strains were at least partly inhibited in cattle previously infected by several other mainland strains As regards the different mainland strains K20 65 strain appeared to be least effectively inhibited by any combination of the other strains J-Puke strain which was also the least virulent of them never resulted in a detectable infection (the possible reaction in heifer 24 excepted (see Table 1)) in cattle with earlier experience of other strains but every animal concerned had previously reacted to some other Juva strain (J34-64 J1-65) with which J-Puke strain appeared to be immunologically more closely related No close immunological relationship was found between the two Kangasniemi strains calf 3 did not react to inoculation with K1-65 strain after experience of K20 65 strain but it had alread had experience of several other strains

Although the resistance to new heterologous strains was gradually augmented by an increasing number of reactions with different strains there was recorded no obvious prolongation of the incubation period comparable with that observed in the two previously described positive challenges by the homologous strain (9) it was suspected that slight prolongation had occasionally taken place

Some interesting items of information were provided by a series of experiments originating from a phase of serial reactions in splenectomized sheep 2

Sheep 2 (see chart in Fig 1) was inoculated with J1 65 strain after about 1 month had passed since reaction to J Puke strain It suffered a rather mild attack Subinoculations of blood were made into three sheep (sheep 7 14 and 16) one of which (sheep 7) had previously experienced J Puke infection Both sheep 14 and 16 had earlier been inoculated with Jo5-65 strain in all tests this had proved to be non virulent in sheep (10) In addition sheep 16 had been inoculated with blood obtained from an animal reacting to J1 65 strain but subsequently stored at +4 C for several days No reaction to this inoculation was remarked The results of the subinoculations of sheep 2 blood were as follows Sheep 7 did not react detectably In 4 days sheep 14 had a mild reaction with only a few tick borne fever bodies observable Subinoculation of its blood into calf 23 (Fig 1) which had previously reacted to J34 64 strain caused a strong reaction After an incubation period of 6 days sheep 16 experienced a strong reaction with a high percentage of infected granulocytes (cf (10) as regards virulence of strains) When the two sheep last mentioned were later challenged with J Puke strain sheep 14 reacted but sheep 16 did not The result is so interpreted that the subinoculation of sheep 2 blood into sheep 14 brought about a reaction by J1 65 strain and in sheep 16 by J Puke strain and that accordingly sheep 2 was a carrier of J Puke strain concurrently with its reacting to J1 65 strain

The most probable explanation for sheep 14 apparently reacting to J1 65 strain and sheep 16 to J Puke strain seems to be as follows Sheep 16 might have reacted very mildly to the preceding inoculation with J1 65 strain but nevertheless developed immunity against it As sheep 2 was a low level carrier of J Puke strain

both strains were probably subinoculated into each sheep In sheep 16 J1-65 strain was inhibited by homologous immunity but by reason of the lack of sufficient cross immunity J-Puhe strain could cause a reaction The dominance of J1-65 strain in the inoculum ensured that within a shorter period it could cause a reaction in sheep 14 than J-Puhe strain would have been capable of (cf incubation periods in the two sheep) and probably suppressed the further development of J-Puhe strain by interference (cf (10))

DISCUSSION

At the present state of knowledge at least the extensive immunological heterogeneity observed in this study among the randomly isolated strains of the tick borne fever agent appears to be unparalleled by any other species of micro organisms In particular the finding that even the sequential inoculation of several heterologous strains each causing a reaction was frequently inadequate to produce effective cross immunity to a new challenge strain evidences immunological heterogeneity of amazing proportions

It is likely that the first questions posed by the reader will be how reliable are the results presented do they really reflect the existence of immunological heterogeneity or could they be explained in other terms? For the proposition that the results express immunological heterogeneity to be true the condition is imposed that the development of solid and durable homologous immunity is the rule That this condition must have been generally fulfilled in the animals subjected to the serial inoculations reported is strongly supported by the findings concerning homologous immunity (9) Most of the series of sequential inoculations were made within a period during which it could be assumed that homologous immunity resulting from the first reaction had remained effective At times challenges by the homologous strain were made during the course of heterologous inoculations (Fig 1 and Table 1) they were invariably negative It thus seems rather convincing that at least the bulk of reactions induced serially by heterologous strains are expressions of real immunological differences

It is obvious that even the homologous immunity in tick borne fever on comparison with that in many other infections is relatively weak experiences from British ovine tick borne fever discussed previously (9) illustrate this relative weakness even better than findings concerning the Finnish strains This fact with the implication that immunity against heterologous strains is bound to be still weaker may account for the easier detection of immunological strain differences of the tick borne fever agent than of strain differences in other species of micro organisms which induce stronger immunity

In regard to rickettsiae rather extensive serological and immunological differences apart from those in virulence are known to occur between strains of *Rickettsia tsutsugamushi* (reviewed by *Smadel & Elisberg* (6)) It is interesting that the immunological differences in cross immunity tests were detected only when immunization was ef

in Tables 2 and 3 The figures in parentheses indicate that this kind of lack of protection was encountered very frequently

If Fig 1 and Table 1 are further examined, it is discovered that infection by J-Puhe and K20-65 strains was never full inhibited in sheep by virtue of previous experience of any combination of heterologous strains. Although partial suppression was occasionally indicated as having taken place nevertheless every reaction by these strains was easily detectable In cattle all the inoculations by Jo5-65 strain produced a marked tick borne fever reaction. The impression was gained that even the experience of several mainland strains (see Fig. 1(9) for location of their origin) exercised no more than a slight effect, if an on the course of the infection by this strain which originated from Aland archipelago On the contrary all the mainland strains were at least partly inhibited in cattle previously infected by several other mainland strains. As regards the different mainland strains, K20-65 strain appeared to be least effectively inhibited by any combination of the other strains, J-Puhe strain which was also the least virulent of them never resulted in a detectable infection (the possible reaction in heifer 24 excepted (see Table 1)) in cattle with earlier experience of other strains, but every animal concerned had previously reacted to some other Juva strain (J34-64 J1-65) with which J-Puhe strain appeared to be immunologically more closely related. No close immunological relationship was found between the two Kangasniemi strains calf 3 did not react to inoculation with K1-65 strain after experience of K20-65 strain, but it had already had experience of several other strains.

Although the resistance to new heterologous strains was gradually augmented by an increasing number of reactions with different strains, there was recorded no obvious prolongation of the incubation period comparable with that observed in the two previously described positive challenges by the homologous strain (9) it was suspected that slight prolongation had occasionally taken place

Some interesting items of information were provided by a series of experiments originating from a phase of serial reactions in splenectomized sheep 2

Sheep 2 (see chart in Fig 1) was inoculated with J1-65 strain after about 1 month had passed since reaction to J-Puhe train It suffered a rather mild attack. Subinoculations of blood were made into three sheep (sheep 7 14 and 16) one of which (sheep 7) had previously experienced J-Puhe infection Both sheep 14 and 16 had earlier been inoculated with Jo5-65 strain in all tests this had proved to be non virulent in sheep (10) In addition sheep 16 had been inoculated with blood obtained from an animal reacting to J1-65 train but subsequently stored at -4 C for several days No reaction to this inoculation was remarked. The result of the subinoculations of sheep 2 blood were as follows. Sheep 7 did not react detectably In 4 days sheep 14 had a mild reaction with only a few tick borne fever bodies observable. Subinoculation of its blood into calf 23 (Fig 1) which had previously reacted to J34 64 strain caused a strong reaction After an incubation period of 6 days sheep 16 experienced a strong reaction with a high percentage of infected granulocytes (cf (10) as regards virulence of strains) When the two sheep last mentioned were later challenged with J-Puhe strain sheep 14 reacted but sheep 16 did not. The result is so interpreted that the subinoculation of sheep 2 blood into sheep 14 brought about a reaction by J1-65 strain and in sheep 16 by J-Puhe strain, and that accordingly sheep 2 was a carrier of J-Puhe strain concurrently with its reacting to J1-65 strain

The most probable explanation for sheep 14 apparently reacting to J1-65 strain and sheep 16 to J-Puhe strain seems to be as follows. Sheep 16 might have reacted very mildly to the preceding inoculation with J1-65 strain, but nevertheless developed immunity against it. As sheep 2 was a low level carrier of J-Puhe train

that variants of the tick borne fever agent greater in number than those of *R tsutsugamushi* for example could exist in a limited region However the matter is not so simple as that as is indicated by the finding of *Miesse Diercks & Danauskas*, who isolated strains of *R tsutsugamushi* of four antigenic types from the area of a small field (4) An earlier description (10) indicates that variants of the tick borne fever agent can apparently exist simultaneously on the same pasture This finding serves to make it still more easily comprehensible that no two identical strains were isolated during the present studies

SUMMARY

Eleven strains of tick borne fever agent from different parts of Finland of which all but one had been isolated from bovine field cases were subjected to immunological comparison Cross immunity tests were made in experimental cattle and sheep These tests could be extended to comprise the serial inoculation of animals with several heterologous strains

The strains proved to have great immunological heterogeneity To some extent each strain seemed to differ from every other Three strains which originated in Juva parish appeared to bear a closer mutual relationship than did the others The very extensive nature of the heterogeneity among the strains was demonstrated by the results of serial inoculations with heterologous strains One calf reacted to the inoculation of 7 strains In three calves and two sheep tick borne fever reactions were induced with 5 to 6 heterologous strains although the gradual development of broad cross immunity in the course of serial inoculations was noted No cross immunity was apparent between the Finnish strains and two Scottish ovine strains

Simultaneous infection with two heterologous strains was discovered in a sheep

The extent of immunological heterogeneity in the strains of tick borne fever agent seems unparalleled in any other species of microorganisms given sufficient study The paper concludes with a discussion of the mechanism of immunity in tick borne fever

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Acta path et microbiol scandinav 71 101–108 1967

State Veterinary Medical Institute Helsinki and Department of Virology University of Helsinki Finland

EXPERIMENTAL STUDIES ON BOVINE TICK BORNE FEVER

4 *Immunofluorescent Staining of the Agent and Demonstration of Antigenic Relationship between Strains*

By

JAAKKO TUOMI

Received 27 xii 66

Investigations of tick borne fever have been greatly hampered by the failure to apply serological methods The present paper describes the adaptation of a direct immunofluorescence method for examination of the tick borne fever agent and reports the finding that despite immuno logical differences all the strains studied appear to be related anti genically

MATERIAL AND METHODS

Antisera and their fractionation In 1964–65 eight sera to be labelled with fluoro chrome were obtained from 6 experimental cattle which had experienced reactions by one or more strains of tick borne fever agent A more detailed description of the bovines their reactions and the agent strains concerned has been given in earlier papers of this series (6 7 8) Cow 7 had reacted to J43 64 and Jo3 64 strains the serum was collected one week after reaction by J53 64 Cow 10 had reacted to J34 64 strain and had afterwards been challenged twice with the same strain the serum was obtained 3 months after the reaction (the last challenge had been made two weeks previously) From both heifers 13 and 14 sera were obtained on two occa sions The first sera (I) were taken when about 1½ months had elapsed after reac tion to the J34 64 strain and about two weeks after the homologous challenge of both heifers The second serum (II) from heifer 13 originated from a time about three weeks after the subsequent reaction to K1 65 strain and the second serum (II) from heifer 14 about 3 weeks after reaction to J1 6 strain The serum from heifer 24 was collected after the experience of reactions to Jo5 65 nd J1 65 strains about two weeks after J1 65 Calf 26 had been inoculated (and challenged once) with Jo5 65 strain only and serum was taken about two we ks after the reaction

Sera were fractionated by half saturation with ammonium sulphate as described by *Cherry et al* (1) The dialysed gl bul n f a ti n w used for l belling

Serum from a cow with no experien f ti k borne fe er was treated imila ly for the provision of a control conjugat

Conjugation and purification procedures All the serum globulin samples were conjugated with crystalline fluo e cein isothiocyanate (FITC) (Dajac Laboratories Philadelphia) in accordance with the method presented in the monograph by *Nairn* (editor) (5) but with *some modific t ons* All the globul n solutions except that from cow 10 we e conjugated with yellow coloured flu rescein from the same

This work was supported by grants from the *Finnish Medical Research Coun l* and from the *Ministry of Agriculture*

lot the stain used for conjugation of cow 10 serum originated from a different lot and was brown in colour The protein concentration of the globulin solutions was determined spectrophotometrically by means of the Biuret reaction (?) FITC was added in powder form to an amount which would give a dye protein ratio of 3 mg to 100 mg (4) One half the amount of carbonate bicarbonate buffer had been added to each globulin solution The unreacted FITC was removed by gel filtration on Sephadex G 25 (medium grade) (Pharmacia Uppsala Sweden) The conjugate was eluted with phosphate buffered physiological saline (PBS 0 01 M phosphate pH 7 5) Part of the conjugate was then absorbed twice with acetone dried calf liver powder (5) Merthiolate was added to give a 1 5000 dilution and the conjugate was stored at +4 C

For use in background counterstaining bovine plasma albumin solution (5 per cent) in saline was conjugated with lissamine rhodamine B (RB 200) (George T Gurr Ltd London) the method given in Nairn s monograph (5) being used The conjugate was briefly dialysed against PBS but not absorbed with liver powder

Antigen preparations The antigen preparations were blood smears both from experimental cattle and sheep suffering from tick borne fever and from bovine field cases of the disease occurring in summer 1965 Reactions from which blood smears were made and which were demonstrated as being tick borne fever by the examination of Giemsa stained smears had been caused by 30 different agent strains Of these strains 19 originated from the parish of Juva 3 from Kangasniemi 1 from Tuulos 1 from Asikkala 4 from the Aland archipelago and 2 were Scottish strains of ovine tick borne fever agent Those of the strains which had been used in experimental studies (J34 64 J1 65 J Puhe K1 65 K20 65 Jo5 65 Jo6 65 T1 65 7 C93 and 8 C90) have been described previously (6 7 8)

Both thin and thick blood smears were used The preparation of thick blood smears was done in the way described for malaria parasite examinations (10) Smears were routinely allowed to dry at room temperature for 6 hours to 1 day and then either used or stored at +4 C or —20 C Occasionally smears were made from white cell concentrates The preparation of concentrates has been described previously (9)

Staining procedure Blood smears were routinely fixed in dry acetone for 10 minutes at room temperature the stored smears were allowed to dry at room temperature for half an hour before fixation When staining was effected with the FITC conjugate alone one to two drops were applied to the smear for thin smear this was done to the tail portion where white cells are concentrated Slides were incubated in a moist chamber at room temperature for 45 minutes washed with PBS for 10 minutes (one change of buffer) and mounted in phosphate buffered glycerol (90 per cent pH 8 0)

For counterstaining RB 200 conjugate was diluted 1 10 in PBS and one drop of both FITC conjugate and RB 200 conjugate were applied and mixed together on the smear

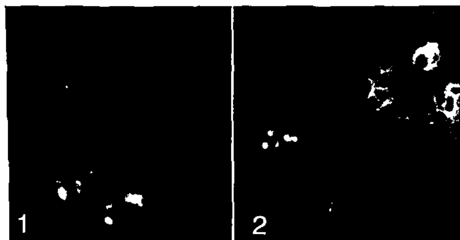
Microscopy A Leitz Ortholux microscope was used The light source was an Osram HBO mercury vapour lamp A bright field UV condensor was employed The objectives were 25x 40 x and 60 x and occasionally 90 x oil immersion

The intensity of the fluorescein fluorescence was graded ++ = brilliant green fluorescence +± = moderate fluorescence + = definite but weak fluorescence ± = doubtful fluorescence

RESULTS

Staining of Bodies in the Cytoplasm of Granulocytes

Several of the FITC conjugates caused the staining of different sizes of body in the antigen preparations (Figs 1 and 2) The size of these green to yellow green fluorescing bodies varied from approximately 0 5 μ up to 4 μ in diameter The close proximity of these bodies to non fluorescing lobed nuclei indicated their location in the cytoplasm of granulocytes Usually the cytoplasm of neutrophil granulocytes had a very faint grey autofluorescence which permitted of the nuclei being



Figs 1 ~

Fig 1 Fluorescent bodies in the cytoplasm of two neutrophils The antigen preparation was a thin film of leukocyte concentrate from calf 4 reacting to T1 65 strain of tick borne fever agent Cow 7 FITC conjugate used for specific staining and RB 200 conjugate for counterstaining × 1200

Fig 2 Fluorescent bodies in the cytoplasm of neutrophils An eosinophil with auto fluorescing cytoplasm is on the right Antigen preparation and conjugates the same as in Fig 1 × 300

discerned The fluorescing bodies were more or less round In intensively stained preparations the larger bodies occasionally displayed an uneven staining with a suggestion of their being composed of fluorescing particles Ringforms with nonfluorescing centres were sometimes observed In weakly stained preparations the smallest bodies were discerned only with difficulty or not at all the staining of larger bodies was more easily detectable

Apart from their presence in cells which were apparently neutrophil granulocytes fluorescing bodies were also observed in cells with gray yellow granular cytoplasm These cells had also lobed nuclei and were considered as representing eosinophil granulocytes (Fig 2) In various preparations their proportionate occurrence in comparison with the cells suggested to be neutrophils corresponded to the proportion found in Giemsa stained smears This finding is in agreement with that made by *Fuerst & Jannach* that the cytoplasm of eosinophils displays auto fluorescence (3)

Both thin and thick blood smears were usable as antigen preparations In thin smears the details of location of the bodies within granulocytes could be studied more closely as each cell was spread over a wider area than that in thick smears Thick smears offered the advantage that more cells were present in a microscopic field and in the event of sparseness of bodies they were found more easily By the utilization of thin smear preparations made from white cell concen

but the stain used for conjugation of cow 10 serum originated from a different lot and was brown in colour The protein concentration of the globulin solutions was determined spectrophotometrically by means of the Biuret reaction (2) FITC was added in powder form to an amount which would give a dye protein ratio of 3 mg to 100 mg (4) One half the amount of carbonate bicarbonate buffer had been added to each globulin solution The unreacted FITC was removed by gel filtration on Sephadex G 25 (medium grade) (Pharmacia Uppsala Sweden) The conjugate was eluted with phosphate buffered physiological saline (PBS 0 01 M phosphate pH 7 5) Part of the conjugate was then absorbed twice with acetone dried calf liver powder (5) Merthiolate was added to give a 1 5000 dilution and the conjugate was stored at +4 C

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Staining procedure Blood smears were routinely fixed in dry acetone for 10 minutes at room temperature the stored smears were allowed to dry at room temperature for half an hour before fixation When staining was effected with the FITC conjugate alone one to two drops were applied to the smear for thin smears this was done to the tail portion where white cells are concentrated Slides were incubated in a moist chamber at room temperature for 45 minutes washed with PBS for 10 minutes (one change of buffer) and mounted in phosphate buffered glycerol (90 per cent pH 8 0)

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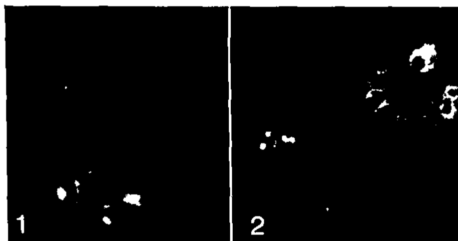
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trates there could be gained the advantages offered by both thin and thick whole blood smears

The cytoplasm of granulocytes was made to fluoresce faintly in red brown colour by counterstaining with RB 200 conjugate The method offered no advantage in the detection of green fluorescing bodies but it enabled better visualization of the location of these bodies in the cytoplasm of granulocytes

No fluorescing bodies were present in preparations made from the blood of healthy cattle or sheep or from the blood of cattle with piroplasmosis or eperythrozoon infection Moreover FITC labelled serum globulins from the cow with no experience of tick borne fever did not stain the bodies Only rarely was anything suggestive of nonspecific staining encountered stain which occasionally lined white cells gathered in larger groups was regarded as representing such staining

Observations on Fixation and Preservation of the Antigen and Preservation of the Conjugate

Acetone fixation of the antigen preparation proved superior to the other methods investigated In air dried preparations subjected to no further form of treatment bodies were usually also stained but the fluorescence was definitely weaker than after acetone fixation Momentary dipping of the preparation into absolute alcohol or treatment for 5 min in 0 3 N hydroxy chloride inhibited the staining of bodies

Smears were either stored unfixed and fixed with acetone immediately before use or were stored in the acetone fixed state Satisfactory staining was achieved in preparations stored for 3–4 days at room temperature Storage at +4 C gradually impaired the stainability of the bodies as a rule definite impairment was observed to have taken place in two weeks Weak staining of bodies was once noted in preparations kept fixed in acetone for 1½ months at +4 C It is possible that the storage of acetone fixed smears was more successful at +4 C than that of unfixed smears Storage at —20 C for 2 months of both unfixed or acetone fixed smears did not detectably affect their stainability No examination was made of the effect of storage for more lengthy periods

One interesting finding was the good preservation of the antigens in EDTA blood stored at +4 C Smears made from several blood samples stored for up to 4 months appeared to display staining of bodies as intensive as those made from fresh blood (The effect of even longer storage was not examined) Although brightly stained most of the bodies exhibited signs of disintegration and the dispersion of small particles over wide areas

FITC conjugates appeared satisfactorily to retain their staining properties at +4 C The conjugate of the serum globulins from cow 7 (cow 7 conjugate) tested after the longest period of storage (1 year

and 2 months) did not exhibit any definite diminution in the intensity of staining brought about

Antigenic Relationship of Strains

Conjugates prepared from sera from different cows or in different phase from the same cow could differ in the intensity of the staining of bodies they caused but no more than minor differences if any were observed in the capability of each conjugate to stain bodies of various agent strains The conjugates of cow 7 heifer 13 (II) and heifer 14 (II) generally induced a bright ++ staining of bodies in properly pre served antigen preparations Heifer 14 (I) and heifer 24 conjugates in duced slightly less intensive staining usually graded +± to ++ Rather weak fluorescence was given by the calf 26 conjugate + to +± Heifer 13 (I) and cow 10 conjugates never resulted in a detectable stain in bodies of the granulocytes

Antigen preparations from 27 of 30 agent strains studied were examined with and all stained by cow 14 (II) conjugate It is possible although not definitely con firmed that the staining of bodies in Jo5 65 and the Scottish ovine strain antigen preparations might have been slightly weaker than in other preparations In the Jo5 65 strain preparations examined the number and the size of bodies were smaller than usual this might have influenced the result However calf 26 conjugate which represented antibodies to Jo5 65 strain appeared to stain bodies caused by the homologous strain as intensively as or slightly more than bodies by the other 7 strains tested

In heifer 14 (II) conjugate antibodies induced by two Juva strains J34 64 and J1 65 were probably present heifer 14 (I) conjugate was made from serum ob tained when the animal had experienced infection by J34 64 strain only The 16 heterologous antigen preparations examined by means of the latter conjugate re presented agent strains from every parish concerned along with the Scottish ovine strains Every well preserved preparation was stained to at least +± intensity in the case of some Juva strains staining may have been somewhat brighter than when the antigen originated from other parishes or was produced by the Scottish strains It was further suggested although not certain that heifer 24 conjugate made after experience of Jo5 65 and J1 65 strains possibly stained the antigen of the homo logous strains to a slightly greater intensity than the antigens of 5 other strains tested Heifer 13 (II) conjugate (15 strain tested) made after the reaction caused by K1 65 strain did not stain the antigen of this strain more intensively than did the conjugates of cow 7 (9 strains tested) and heifer 14 (II)

An examination was made of the staining of antigens of the Scottish strains with the conjugates of cow 7 and heifer 13 (II) Application of these conjugates also resulted in the fluorescence of bodies which was in accord with the pattern produced by each conjugate in regard to other strains although possibly the intensity was again slightly inferior to that induced in the brightest staining antigen preparations of Finnish strains

As was proved by the staining ability of calf 26 conjugate serum obtained 2 weeks after subsidence of the reaction could in this case be used for labelling

DISCUSSION

The confinement of fluorescein fluorescence to bodies resembling Giemsa stained tick borne fever bodies in form and size and located in the cytoplasm of granulocytes strongly supports the conception that

in fact the structures stained were tick borne fever bodies That the attachment of fluorescein to tick borne fever bodies was effected through antigen antibody reaction is indicated by the general absence of staining of any other structures or normal cell constituents and by the inability of the control conjugate to stain these bodies A preliminary finding has been made that the staining of bodies is reduced by prior treatment with unlabelled immune serum and that bodies can be stained by application of the indirect immunofluorescence method (*Tuomi* unpublished data)

The finding that the staining in some bodies seemed to be confined to smaller particles and the observation of ring forms with unstained centres suggest that the labelled immunoglobulins were attached to the agent particles themselves and not to other constituents of the phagocytic vacuoles containing these particles (for fine structure of tick borne fever bodies cf (9))

It consequently seems obvious that the immunofluorescence technique offers a greatly needed serological means for further studies of tick borne fever Pertinent subjects for such studies include the occurrence and significance of antibodies in tick borne fever the antigenic structure of the causative agent and the possible antigenic relationship it may bear to other species of microorganisms

The finding that heifer 14 (I) and calf 26 conjugates each representing antibodies to a single strain of tick borne fever agent stained all the heterologous strains examined (heifer 14 (I) conjugate even the Scottish ovine strains) indicates that various strains of the agent share a common antigen or antigens a result by no means unexpected However it was slightly surprising that all the heterologous strains were stained at least nearly as effectively as was the homologous strain particularly in view of the demonstration that very wide immunological differences may exist between strains (8) The result is interpreted to suggest that a common antigen (or antigens) is a regular constituent or product and one which occurs rather abundantly of the agent particle but one that does not seem effectively involved in the development of immunity against the agent It may well be that if more refined techniques are applied including dilution and absorption with heterologous strains of the conjugates and use of the indirect method then antigenic differences between immunologically deviating strains can be discovered and investigated further

There appeared to exist a rough correlation between the extent of experience of different strains by the bovine from which the conjugated globulins originated and the staining intensity of the conjugate This probably implies that the titer of the antibodies to the common antigen (antigens) is increased by the serial inoculation of heterologous strains The staining ability of calf 26 conjugate indicates that these antibodies are already present at least in small amounts two weeks after subsidence of the first tick borne fever reaction

It remains obscure why cow 10 and heifer 13 (I) conjugates did not stain tick borne fever bodies at all There is no knowledge whether the lot of FITC used for the conjugation of cow 10 globulins might have deteriorated but this is perhaps possible Alternatively the antibody level in cow 10 might have substantially fallen in the three months which had elapsed since the reaction despite the two challenges with the homologous strain Even greater obscurity is attached to the lack of staining by the heifer 13 (I) conjugate particularly as the heifer 14 (I) conjugate induced rather intensive staining and both animals had almost identical histories of experience of tick borne fever Bright staining resulting from heifer 13 (II) conjugate demonstrates that this heifer also was well capable of producing antibodies The most probable explanation may be unremarked technical error in the preparation or conjugation procedures or rapid deterioration of the conjugate during storage

The discovery that the antigen is well preserved in and is easily detectable from blood stored at +4 C for up to 4 months provides not only theoretical interest but a practical method of diagnosis in cases when blood samples are too poorly preserved to allow of the demonstration of tick borne fever bodies in Giemsa stained smears

SUMMARY

A direct immunofluorescence method has been developed for studies of tick borne fever It was demonstrated that six samples of immuno globulins labelled with fluorescein isothiocyanate and coming from 5 experimental cattle with varying experience of strains of the tick borne fever agent stained tick borne fever bodies present in blood granulo cytes Both thin and thick blood smears could be utilized as antigen preparations Fixation was effected with acetone Counterstaining with lissamine rhodamine B conjugate enabled better visualisation of con finement of the specific fluorescence to the cytoplasm of granulocytes

Tick borne fever bodies in preparations made from EDTA blood stored at +4 C for periods of up to four months were stained to degrees of intensity which were practically equal to those obtained with preparations from fresh blood

Studies were made of the staining of 28 agent strains all but one of them isolated from bovine field cases originating from 5 different regions of Finland and 2 Scottish agent strains of ovine tick borne fever Every strain tested was stained by each of the 6 conjugates to an intensity which was roughly characteristic of the particular conjugate On a few occasions it was suspected that more intensive staining of the homologous strain had occurred but this was not certain The Scot tish strains were stained at least nearly as brightly as the Finnish ones It appeared that the staining intensity of the conjugate was correlated with the extent of the donor s experience of tick borne fev

Drugs The effect of the following compounds on the tick borne fever agent was investigated Sodium penicillin C (Na Penita Laake Oy Turku Finland) procain penicillin C (Procain Penita Laake Oy) streptomycin (Bimycin which contains streptomycin and dihydro streptomycin aa Laake Oy) oxytetracycline (Terramycin Pfizer) chloramphenicol (Leukomycin Bayer) sulphamezathine (Sulfatin Laake Oy) The last drug was injected intravenously all the others intramuscularly In addition sulphamezathine powder was received from Orion Oy Helsinki for oral administration in one experiment

Strains of agent The origin and properties of the three strains used in the present experiments J34 64 J1–65 and K9–66 have been described earlier (15 16 17) In each experiment the animals concerned were inoculated intravenously with 1 ml of infective blood

TABLE 1

Tests on Sensitivity of J34 64 Strain of Tick Borne Fever Agent to Various Drugs

Test animal	Drug applied	Treated daily for five days starting three days after inoculation of agent	Treated daily for three days starting three hours before inoculation of agent	Daily dose	Reaction after incubation period in days
Heifer 18	Proc penic	×		1 5 milj I E / 100 kg b	7
Cow 19		×			7
Cow 15	Streptom	×		1 5 g/100 kg	7
Heifer 20		×			7
Cow 16	Oxytetra cycline	×		0 5 g/100 kg	no reaction
Cow 22			×		no reaction
Cow 21	*Sulpha mezathine*		×	*10 g/100 kg*	*no reaction*
Cow 17	No treatment				7

a = refers to first appearance of tick borne fever bodies
b = in connection with the first treatment also sodium penicillin C 0 7 milj I E /100 k was applied
c = additional injection (2/3 of the normal dose) 4 hours after inoculation of the agent

RESULTS

In the first experiment set out in detail in Table 1 the effect of penicillin streptomycin oxytetracycline and sulphamezathine on the tick borne fever agent was investigated The agent proved sensitive to both oxytetracycline and sulphamezathine but neither penicillin nor streptomycin appeared to have any effect on it under the conditions of the experiments The temperatures of the three cows which failed to react to the inoculated agent were observed for more than one month but no signs of illness ensued When cow 16 was challenged with the same agent strain six months later it reacted like a fully susceptible animal

In another experiment the inhibitory effect of oral application of sul phamezathine powder was studied Two cows (cows 34 and 35) re ceived 5 doses (20 g/100 kg) of sulphamezathine at intervals of five days Three days after the first dose J1 65 strain was inoculated into these two cows and one control heifer (heifer 36) The heifer suffered a tick borne fever attack starting 6 days after the inoculation No re action appeared in cows 34 and 35 whose temperatures were followed 1½ months after inoculation of the agent even subsequently no clinic al signs of illness were recorded

In the third experiment the sensitivity of the tick borne fever agent to chloramphenicol was tested In the first part sheep 22 and 24 were inoculated with K9-66 strain immediately after the administration of chloramphenicol to sheep 24 at a dose of 0 4 g/10 kg This sheep re ceived three additional equal doses of the drug 18 42 and 50 hours after the inoculation Both sheep reacted to inoculation the incubation period in sheep 22 was 5 days and in sheep 24 6 days

In the second part of the experiment with sheep 4 and 15 the aim was a more effective treatment with cloramphenicol Two hours after its administration to sheep 15 in a dose of 0 4 g/10 kg both animals were inoculated with K9 66 strain Five hours after this inoculation sheep 15 received a second similar dose of chloramphenicol which was furthermore repeated twice on the two following days and once on the day thereafter Both sheep again experienced a tick borne fever reaction but the incubation period was clearly prolonged in sheep 15 the reac tion commenced five days and nine days after the inoculation in sheep 4 and 15 respectively The tick borne fever reactions were about equal in severity in all four sheep

DISCUSSION

The suggestion derived from treatment of field cases (19 12 4 14) that bovine and ovine tick borne fever agents are sensitive to oxytetra cycline is supported by the results of the present study *Øveras* (12) observed that after treatment with oxytetracycline one sheep experi enced a spontaneous relapse and that other sheep treated could remain carriers of the agent It is indicated by his paper that as a rule only one dose of oxytetracycline was given In cow 16 of the present study which was treated with oxytetracycline daily for five days tick borne fever infection in its initial phase was not only suppressed but apparently also abolished This finding suggests that the tick borne fever agent may be eliminated from the host by protracted treatment with oxytetracycline Findings in agreement with those presented above as regards the ef fect of tetracycline compounds have been made *e g* in connection with infections by the psittacosis group of agents (7 8) with which organ isms the tick borne fever agent has certain similarities (18)

Further support of the contention that oxytetracycline therapy may

be more than static with regard to tick borne fever infection is furnished by the recorded failure of the agent to become established in cow 22. Establishment of infection was also prevented by intravenously and orally administered sulphamezathine. A parallel observation by Foggie (3) in respect of the effect of sulphamezathine was cited in the introduction. However, Foggie could not eliminate an already established tick borne fever infection in sheep by oral treatment with sulphamezathine. A question of mainly theoretical interest is whether elimination may be accomplished by prolonged effective sulphamezathine therapy and whether there are differences in effectivity between tetracyclines and sulphonamides in this regard.

It is further suggested by the results of the present study that the tick borne fever agent is sensitive to chloramphenicol but that the effect of this drug might be inferior to that of oxytetracycline and sulphamezathine. There is some evidence that chloramphenicol may be less effective than tetracyclines against *Coxiella burneti* (reviewed by Ormsbee (11)), another remote relative of the tick borne fever agent (18).

In connection with an electron microscopic study of the tick borne fever agent, its probable taxonomic position in the middle ground between typical rickettsiae and the organisms of the psittacosis-lymphogranuloma-trachoma (PLT) group was discussed (18). In sensitivity to sulphonamides the tick borne fever agent differs from typical rickettsiae and is related to some representatives of the PLT group. On the other hand, the present results indicate that the tick borne fever agent is either totally insensitive or has a very low sensitivity to penicillin, a property which it shares with rickettsiae but not with the PLT group (9).

Among the causative agents of other animal rickettsioses, *Cowdria ruminantium* has been reported to be sensitive to sulphonamide chemotherapy (10) and to tetracyclines (5). The existence of morphological similarities between this organism and the PLT group has been pointed out (13). It remains to be clarified by further study whether the three representatives of the genus *Ehrlichia* first described by Donatien & Lestoquard (1, 2) and by Lestoquard & Donatien (6), which multiply in the blood monocytes of dog, cattle and sheep respectively, and which show some morphological similarities with the PLT group and the tick borne fever agent, too, are closer to the latter in their sensitivity to antibiotics and sulphonamides than to typical rickettsiae.

SUMMARY

Daily treatment with oxytetracycline during 5 days inhibited the development of and apparently eliminated the infection by tick borne fever agent inoculated 3 days prior to commencement of the treatment. Both oxytetracycline and sulphamezathine, each in one cow, prevented the establishment of the agent when parenteral treatment was started shortly before inoculation and kept up during two more days. Estab-

lishment of infection was likewise inhibited in two cows by orally ad ministered sulphamezathine Chloramphenicol given to two sheep under the conditions of the present experiments neither prevented the estab lishment of infection nor eliminated the agent but it appeared to sup press the multiplication of the agent to judge from the prolonged in cubation period observed

Penicillin and streptomycin treatment did not seem to have any in hibitory effect on the development of the infection

The taxonomic implications of the drug sensitivity of the tick borne fever agent are discussed

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uniform than in other similar experiments because of a technical defect in the ultrasonic apparatus. The only vaccine which was fairly well dispersed was Dubos Chicago; Gothenburg was the most lumpy one among the four vaccines as determined by differential counts and it also contained the lowest number of units per ml.

TABLE 1

Number of Viable Bacterial Units and Bacteria in 1 ml of 10 BCG Suspension before (—) and after (+) Ultrasonic Treatment

BCG strain	No. of units per ml		No. of bacteria	
	—	+	per unit +	per ml +
Dubos Copenhagen	13.6×10^{6}	34.0×10^{6}	2.6	8.8×10^{7}
Rio de Janeiro	9.8×10^{6}	16.4×10^{6}	3.9	5.4×10
Gothenburg	1.7×10^{6}	7.6×10^{6}	4.9	3.7×10^{7}
Dubos Chicago	3.2×10^{6}	41.1×10^{6}	1.6	6.6×10

Vaccination. Vaccination of the guinea pigs was performed over two days, on the first day with the strains that on the basis of previous hamster experiments were deemed to be the weakest, i.e. Dubos Chicago and Gothenburg, and on the second day those presumed to be the strongest, i.e. Rio de Janeiro and Dubos Copenhagen. A dose of 0.1 ml of vaccine was applied intracutaneously into the middle of the lower part of the abdomen. First the 10^{-6} dilution of both vaccines was injected and then the 10 and so on. The control animals were given 0.1 ml of the diluent. The vaccination process lasted about 2½ hours. After the guinea pigs were vaccinated 1 ml of the undiluted ultrasonic treated suspension was injected intraperitoneally into the hamsters under ether anaesthetic. Preparation of the vaccines, control inoculation and injection of the animals were carried out by artificial light.

Vaccination time. The interval between the first vaccination day and the first challenge day was 28 days.

Tuberculin testing. Two days before challenge the animals were given two intradermal injections of tuberculin in doses of 0.1 ml, one on each side of the upper part of the back. The dose was 250 TU (3.3 μg) per 0.1 ml of RT 23 with 0.005 per cent Tween 80 prepared at Statens Seruminstitut. The reactions were read after 24 hours by measuring the shortest and longest diameters of erythema. The animals were taken cagewise in random order so that the reader had no knowledge of the experimental group to which they belonged.

Challenge. A culture of *M. bovis* strain E 6884B cultivated as third passage in Dubos fluid medium with Tween was used for challenge of the vaccinated guinea pigs and two non-vaccinated control groups. A 12 day old culture was shaken until it was macroscopically homogeneous, subjected to ultrasonic treatment for ten minutes in the same way as the vaccines and then diluted to 10 with diluted Sauton medium. The bacterial suspension was prepared the day before the challenge. The guinea pigs were injected cagewise in random order and given 0.2 ml of the suspension intravenously into an ear vein. The challenge process was performed on two days and covered in total about 7½ hours. On both days suitable dilutions of the bacterial suspension were inoculated on Dubos oleic acid albumin agar plates and Lowenstein-Jensen tubes. The content of viable units in the suspension was determined by colony counts on the Lowenstein-Jensen tubes, since culture on the Dubos medium gave no growth. On the first and second challenge days the dose per guinea pig contained 105 and 93 viable units respectively. As in the case of the vaccines the bacterial suspension was protected against daylight during preparation and use and was kept in the refrigerator overnight.

On account of the mechanical defect in the ultrasonic apparatus the suspension of the virulent bacteria was, as in the case of the vaccine suspensions, not so well dispersed as in previous experiments. The number of bacteria per unit determined by differential counts on Ziehl-Neelsen stained smears was 6.0 in the untreated culture and 3.5 in the ultrasonic treated culture. Correspondingly it was found by culture that 1 ml of undiluted untreated culture contained 20.9×10^{6} viable units

the treated culture containing 39 3 × 10^9 As the result of the poor effect of the ultra sonic exposure the animals were challenged with about a five times less number of viable units than intended and their survival times were somewhat longer than anticipated

Recording of results After challenge the guinea pigs were observed until they died spontaneously The degree of tuberculosis in the individual animals found at post mortem examination was recorded as Tub 0 to Tub V (*Jespersen et al* 1967 a) The survival times after challenge of the guinea pigs showing moderate or severe tuber culosis at autopsy (Tub IV and Tub V) were used as indication of the acquired resistance The hamsters were also observed until the time of their spontaneous death and the survival times recorded The experiment was concluded nine months after injection of the BCG and animals still surviving were killed The number of tubercle bacilli in the organs was determined by microscopy of smears from liver spleen and lung

RESULTS

Colony morphology The colonies on Lowenstein Jensen tubes could be divided into three types spreading umbilicate and intermediate (*Middlebrook et al* 1947 *Suter & Dubos* 1951 *Froman et al* 1955) Table 2 shows the results of differential counting of 200 colonies for each BCG strain The Dubos Copenhagen strain contained almost ex clusively spreading and no umbilicate types Dubos Chicago₁ contained almost no spreading and many umbilicate types Findings in the Rio de Janeiro and Gothenburg strains ranged in between

TABLE 2

Differential Counting of Colony Types in BCG Suspensions Grown on Löwenstein-Jensen Medium

BCG strain	Type of colony		
	spreading	umbilicate	intermediate
Dubos Copenhagen	96	0	4
Rio de Janeiro	37	15	48
Gothenburg	14	3	83
Dubos Chicago$_1$	8	23	69

Virulence determination on hamsters Among the 20 control animals not injected with BCG only two died (on the 144th and 175th days) during the 9 month experimental period Thus deaths in the BCG in jected groups must in the main be due to the effect of BCG Fig 1 shows the distribution according t[illegible] su[illegible] [illegible]imes of the hamsters inoculated intraperitoneally with [illegible] culture of the four BCG strains The Dubos Copenhagen and Rio de Janeiro strains had the strongest lethal effect on the hamsters Gothenburg came next and last the Dubos Chicago strain A further indication that the Dubos Chicago$_1$ strain is the weakest is that in contrast to the other strains it did not kill the male animals but only the females which are more sensitive to BCG than the males (*Jespersen & Bentzon* 1964 a)

The quantity of tubercle bacilli in liver spleen and lung determined

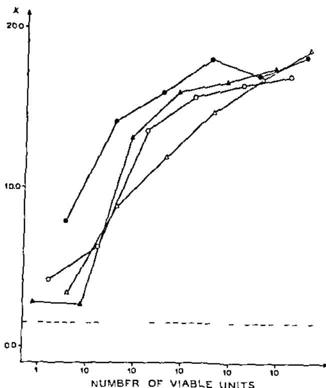


Fig 2

Relationship between mean size of tuberculin reaction ($\bar{x}$ in mm) and number of viable units

● Dubos Copenhagen
○ Rio de Janeiro
▲ Gothenburg
△ Dubos Chicago$_1$
– –– Non vaccinated controls

Evaluated on the basis of the effect on hamsters the Dubos Copenhagen strain was definitely found to be the strongest and Dubos Chicago$_1$ just as definitely the weakest The Rio de Janeiro was stronger than the Gothenburg strain judged by the lethal effect while the difference between the two strains was less marked if considered on the basis of recovery of bacteria or on the macroscopically visible lesions

Tuberculin sensitivity of guinea pigs Table 4 shows the number of animals which became tuberculin positive after vaccination with the various doses and strains 8 mm was chosen as the borderline between positive and negative so that the animals with reactions $\geqq 8$ *mm* were regarded as positive That limit is determined as the least integer greater than the average reaction in the control group plus three times the standard deviation viz $1\ 5 + 3 \times 2\ 1 = 7\ 8$ With the lowest dose only the Dubos Copenhagen strain was significantly different from the control group With the second lowest dose all except the Gothenburg strain differed significantly from the control group The ten times larger dose caused all the animals to become positive except for one

guinea pig vaccinated with the Gothenburg strain and two with Dubos Chicago$_1$

The relative potency of the strains can be evaluated on the basis of the mean size of the tuberculin reactions (Table 4) in guinea pigs vaccinated with the various doses The relation between the tuberculin reactions and the vaccination dose in terms of number of viable units is described graphically in Fig 2 The horizontal distance between curves for the two Dubos strains appears to be constant The average distance graphically estimated corresponds to a dose ratio of 25 indicating that the potency of the Dubos Copenhagen strain is 25 times higher than that of the Dubos Chicago$_1$ The potencies of the two other strains are within the same range This applies to the range of doses

TABLE 5

Statistical Analysis of the Survival Times after Challenge of Guinea Pigs

See Appendix Table

BCG strain	Vaccination dose	No of viable units	No of animals n	Arithmetic mean of reciprocal survival times $\bar{x}$	Standard deviation s_y	Median survival time (in days) $1/\bar{x}$
Dubos Copenhagen	0 1 ml 10^{-6}	3 4	20	0 0078	0 0014	128
	0 1 ml 10^{5}	34	20	0 0077	0 0018	130
	0 1 ml 10^{4}	34 × 10^{1}	20	0 007[illegible]	0 0014	132
	0 1 ml 10^{3}	34 × 10	[illegible]0	0 0070	0 0011	143
	0 1 ml 10 [illegible]	34 × 10^{3}	20	0 0067	0 0016	150
	0 1 ml 10^{1}	34 × 10^{4}	20	0 0067	0 0013	149
Rio de Janeiro	0 1 ml 10	1[illegible]	20	0 0088	0 0016	113
	0 1 ml 10^{5}	16	20	0 0085	0 0016	118
	0 1 ml 10^{4}	16 × 10^{1}	20	0 0079	0 0015	1[illegible]7
	0 1 ml 10^{3}	16 × 10	20	0 0078	0 0016	128
	0 1 ml 10 [illegible]	16 × 10^{3}	20	0 0078	0 0013	129
	0 1 ml 10^{1}	16 × 10^{4}	[illegible]0	0 0073	0 0010	136
Gothenburg	0 1 ml 10^{6}	0 8	20	0 00[illegible]	0 0013	109
	0 1 ml 10^{5}	8	20	0 00[illegible]0	0 0015	111
	0 1 ml 10^{4}	8 × 10^{1}	[illegible]0	0 00[illegible]6	0 0017	13[illegible]
	0 1 ml 10^{3}	8 × 10	[illegible]0	[illegible]	0 001[illegible]	129
	0 1 ml 10	8 × 10^{3}	[illegible]	0 0079	[illegible]0016	1[illegible]7
	0 1 ml 10^{1}	8 × [illegible]	[illegible]	[illegible] 3	0 0015	137
Dubos Chicago$_1$	0 1 ml 10	4	[illegible]	[illegible]087	0 001[illegible]	115
	0 1 ml 10	1	[illegible]	[illegible]0034	0 0018	119
	0 1 ml 10^{4}	41 [illegible] 10^{1}	[illegible]	[illegible]0091	0 0014	109
	0 1 ml 10^{3}	41 × 10	[illegible]0	[illegible]	0 0014	1[illegible]4
	0 1 ml 10	[illegible]1 [illegible] 10^{3}	20	[illegible]081	0 0013	1[illegible]3
	0 1 ml 10^{1}	41 × 10^{4}	[illegible]0	0 00[illegible]6	0 0015	13[illegible]
Non vaccinated controls 1			20	0 009	0 0018	104
Non vaccinated control			20	0 0092	0 0014	10[illegible]
Total			40	0 0094	0 0016	[illegible]

DISCUSSION

Relationship between multiplication of BCG in vivo and extent of acquired resistance In experiments on white mice *Dubos, Pierce & Schaefer* (1953) demonstrated that a strain of BCG which multiplied strongly in the animals (Philadelphia strain) produced a stronger protection against tuberculosis than one which multiplied less strongly (Chicago$_1$) A similar conclusion can be drawn from the experiments carried out by *Willis et al* (1960) with the same strains on guinea pigs

In a previous work *Jespersen & Bentzon* (1964 a) studied the lethal effect on hamsters of 13 BCG strains cultivated in Dubos fluid medium with Tween and injected in large doses applied intraperitoneally On the basis of the survival times of the animals the strains were classified as *strong moderately strong and weak A direct relationship between* the lethal potentiality of the individual strains and their capacity for multiplying in the animals was found Subsequently experiments with eight of these strains (*Jespersen & Bentzon* 1964 b & c) showed a positive correlation between the lethal potentiality of a BCG strain on hamsters and the extent of the acquired resistance induced in red mice These relationships have been confirmed by the present study using *hamsters and guinea pigs*

Relationship between tuberculin sensitivity and acquired resistance in guinea pigs This aspect has been examined previously by *Willis et al* (1960) in a period between the 6th and 30th month after vaccination *i e* in a period when the tuberculin sensitivity and acquired resistance are on the decline after having reached their maximum Guinea pigs vaccinated with the weakly virulent Chicago$_1$ strain were found to lose *their tuberculin sensitivity and acquired resistance more rapidly than* guinea pigs vaccinated with the strongly virulent Philadelphia strain

In cooperation with WHO the BCG Department of this Institute (1965) has examined the same problem using a vaccination period of three to four months Groups of six guinea pigs were vaccinated intradermally with six doses of eight different BCG vaccines containing 1 to 1000 viable units Tuberculin testing with 10 TU RT 23 was performed 12 weeks later *It can be seen from the report of the study that* a Russian and a French vaccine possessed a high allergenic potency while a Czechoslovakian vaccine had little or no effect The potency of other vaccines including that of the Danish and the Japanese 172 were in between In spite of these differences all the vaccines produced almost the same protection against challenge 16 weeks after vaccination with about 1500 viable units of a virulent strain of *M tuberculosis* administered intraperitoneally *The acquired resistance* was evaluated six weeks after challenge on the basis of weight of spleen of the animals The writers concluded that the results seem to show that the allergenic effect of a vaccine does not necessarily indicate its immunogenic effect probably because of differences between the BCG strains

The aim of the present study was to examine whether it might be possible to demonstrate a positive relationship between the tuberculin sensitivity and the acquired resistance in guinea pigs vaccinated with BCG strains of different virulence in the early post vaccination period when the effect of vaccination was under development. It has been demonstrated clearly that the tuberculin sensitivity produced by the Dubos Copenhagen strain within four weeks was higher and the acquired resistance was stronger than that obtained by the Dubos Chicago, strain. The Gothenburg and Rio de Janeiro strains were found to occupy intermediate levels in both respects. Under these experimental conditions the allergenic potency of a strain can be used as indication of its immunogenic potency. This applies only to small or moderately large doses of vaccine. If doses containing more than 10 000 viable units were used it was not possible to determine whether or not strains were attenuated. Neither are the very small doses suitable particularly not if the degree of dispersion varies.

In the present work it seemed to be possible to eliminate the differences in potency of strains if comparatively higher doses of the weak strains were used but in order to compensate for the difference between the weakest and the strongest the dose had to be increased and be about 25 times as high.

It will be seen from the experiment that differences between the allergenic potency of strains were more readily demonstrable than differences in immunogenic potency. This feature is of practical significance and it should thus be of advantage to use the tuberculin test in the control of BCG vaccine. A reduction in the immunogenic potency of the vaccine must be assumed to be accompanied by a considerable reduction in its allergenic capacity. This capacity could be demonstrated by means of one or two suitable doses.

The reason why results obtained in our experiments and those obtained in experiments carried out in the BCG Department are conflicting is probably mainly that the choice of vaccine doses as well as the methods used for an evaluation of the acquired resistance have been different.

As mentioned above, previous experiments have shown that vaccination of guinea pigs with small doses of Danish BCG vaccine involved either that the tuberculin sensitivity as well [illegible] the acqu[illegible]d resistance were present in the individual animals during [illegible]ly post vaccination period or else that neither of these character[illegible] could be demonstrated. This was also the case in the present study in the group of animals vaccinated with the lowest dose of the Dubos Copenhagen strain. Here the median survival time of 11 tuberculin positive animals was significantly prolonged whereas the median survival time of 9 tuberculin negative animals was the same as that of the control animals. The reason why it proved impossible to demonstrate a corresponding relationship as regards the other strains is that equally [illegible]

APPENDIX TABLE

Survival Time (in Days) after Challenge and Size of Tuberculin Reactions (in mm) at Time of Challenge of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with Various Doses of BCG Strains Grown in Dubos Medium

Non vacc non inf controls	Non vacc controls				BCG strain	Vaccination dose and number of viable units											
						0.1 ml 10^{-6} 3.4		0.1 ml 10^{-5} 34		0.1 ml 10^{-4} 34×10^{1}		0.1 ml 10^{-3} 34×10^{2}		0.1 ml 10^{-2} 34×10^{3}		0.1 ml 10^{-1} 34×10^{4}	
days	TR	days	TR	days		TR	days	TR	days	TR	days	TR	days	TR	days	TR	days
223	3	82	0	86	Dubos Copenhagen	0	99	13	85	14	92	18	109	15	104	16	107
244	0	87	5	92		0	105	7	98	17	102	20	109	19	107	21	115
244	3	89	3	93		0	106	11	99	15	111	20	116	14	114	18	127
244	0	91	0	95		8	107	18	106	19	113	21	129	19	121	16	129
244	0	91	5	97		0	110	14	108	16	115	16	134	16	124	20	129
244	0	94	0	99		6	112	11	114	15	124	17	136	16	133	18	130
244	0	95	5	100		16	119	12	115	17	125	19	137	19	140	17	132
244	3	96	6	102		3	120	17	123	20	132	17	140	18	140	20	133
244	0	96	0	105		2	120	18	130	16	135	18	142	17	144	17	136
244	0	97	0	107		10	127	17	135	19	135	18	143	14	146	22	140
244	2	100	5	108		13	128	8	139	15	137	17	144	17	150	16	145
244	0	103	0	109		5	129	20	141	13	138	16	146	18	156	19	154
244	0	107	7	114		7	141	12	143	18	138	18	149	17	158	16	162
244	[illegible]	107	0	117		14	141	15	144	15	140	19	151	19	171	19	165
244	0	108	3	[illegible]		10	143	16	150	17	141	18	155	15	173	17	172
244	0	124	0	123		13	145	13	166	16	147	18	161	19	176	19	173
244	0	130	2	131		9	148	19	168	17	148	17	164	16	207	18	189
244	3	143	0	132		15	165	19	171	15	153	19	172	21	219	20	195
244	0	160	0	[illegible]		14	179	9	177	15	[illegible]	19	178	19	233	19	209
244	0	173	7	168		14	187	17	196	14	228	19	189	18	240	22	214

= killed

APPENDIX TABLE (cont.)

Survival Time (in Days) after Challenge and Size of Tuberculin Reactions (in mm) at Time of Challenge of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with Various Doses of BCG Strains Grown in Dubos Medium

Non vacc. non inf. controls	Non vacc. controls				BCG strain	Vaccination dose and number of viable units											
						0.1 ml 10⁻⁶ 1.6		0.1 ml 10⁻⁵ 16		0.1 ml 10⁻⁴ 16 × 10¹		0.1 ml 10⁻³ 16 × 10		0.1 ml 10⁻² 16 × 10³		0.1 ml 10⁻¹ 16 × 10⁴	
days	TR	days	TR	days		TR	days	TR	days	TR	days	TR	days	TR	days	TR	days
—	3	92	0	96		8	80	13	84	14	101	18	95	16	95	16	106
44		3	5	92		0	85	12	94	12	103	15	96	20	106	14	110
244			3	93		0	95	9	98	14	103	13	107	19	108	17	124
244	0		0	95		6	96	0	102	11	106	15	107	12	110	19	126
244	0	[illegible]		97		0	100	0	104	13	106	17	108	16	112	18	128
44	0	34	3	99		6	106	5	104	12	111	16	112	15	115	17	128
244	0	35	5	100		0	107	0	107	14	115	15	118	17	116	17	129
44		[illegible]		102		0	109	11	108	16	118	13	119	17	116	15	129
	6	6	0	105		3	110	11	110	13	119	15	119	16	1[illegible]3	15	133
44	0		0	107	Rio de Janeiro	[illegible]	114	7	111	15	1[illegible]	18	122	18	125	13	134
44		66		108		12	114	3	115	13	125	18	125	18	125	20	135
244	[illegible]		[illegible]	109		5	115	11	127	15	129	15	149	19	132	20	136
244	[illegible]			114		5	121	6	127	15	132	20	144	15	151	17	138
244	2	107	0	117		3	124	12	130	16	132	16	146	18	151	17	139
244	0	108	3	12[illegible]		0	1[illegible]5	6	132	11	144	14	150	16	152	15	143
244	0	124	0	[illegible]		12	132	16	132	18	152	15	154	15	153	17	151
244	0	130	2	131		7	141	0	141	11	159	13	165	18	155	20	152
244	3	143	0	132		7	144	6	146	12	167	17	157	16	156	18	154
44	0	160	0	165		[illegible]	152	5	174	14	173	15	165	16	159	20	159
244	0	173	7	168		[illegible]	154	3	176	14	194	18	211	16	176	13	197

— killed

APPENDIX TABLE

Survival Time (in Days) after Challenge and Size of Tuberculin Reactions (in mm) at Time of Challenge of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with Various Doses of BCG Strains Grown in Dubos Medium

Non vacc non inf controls days	Non vacc controls				BCG strain	Vaccination dose and number of viable units											
						0 1 ml 10 6 3 4		0 1 ml 10 5 34		0 1 ml 10 4 34 × 101		0 1 ml 10 3 34 × 10		0 1 ml 10 - 34 × 103		0 1 ml 10 1 34 × 104	
	TR	days	TR	days		TR	days	TR	days	TR	days	TR	days	TR	days	TR	days
223	3	82	0	86	Dubos Copenhagen	0	99	13	85	14	92	18	109	15	104	16	107
244	0	87	5	99		0	105	7	98	17	102	20	109	19	107	21	115
244	3	88	3	93		0	106	11	99	15	111	20	116	14	114	18	12[illegible]
244	0	91	0	95		8	107	18	106	19	113	21	129	19	121	16	129
244	0	91	5	97		0	110	14	108	16	115	16	134	16	124	20	129
244	0	94	0	99		6	112	11	114	15	124	17	136	16	133	18	130
244	0	95	5	100		16	119	12	115	17	125	19	137	19	140	17	132
244	3	96	6	102		3	120	17	129	20	132	17	140	18	140	20	133
244	0	96	0	105		2	120	18	130	16	135	18	142	17	144	17	136
244	0	97	0	107		10	127	17	135	19	135	18	143	14	146	22	140
244	2	100	5	108		13	128	8	139	15	137	17	144	17	150	16	145
244	0	103	0	109		5	129	20	141	13	138	16	146	18	156	19	154
244	0	107	7	114		7	141	12	143	18	136	18	149	17	158	16	162
244	2	107	0	117		14	141	15	144	15	140	19	151	19	171	19	165
244	0	108	3	12[illegible]		10	143	16	150	17	141	18	155	15	173	17	172
244	0	124	0	129		13	145	13	166	16	147	18	161	19	176	19	173
244	0	130	2	131		9	148	18	168	17	148	17	164	16	207	18	189
244	3	143	0	13[illegible]		15	165	19	171	15	153	19	172	21	219	20	195
244	0	160	0	13[illegible]		14	179	9	177	15	155	19	178	19	233	19	209
244	0	173	7	168		14	187	17	196	14	228	19	189	18	240	22	214

= killed

APPENDIX TABLE (cont)

Survival Time (in Days) after Challenge and Size of Tuberculin Reactions (in mm) at Time of Challenge of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with Various Doses of BCG Strains Grown in Dubos Medium

Non vacc non inf controls	Non vacc controls				BCG strain	Vaccination dose and number of viable units									
						0 1 ml 10 6 4 1		0 1 ml 10 5 41		0 1 ml 10 4 41 × 10¹		0 1 ml 10 3 41 × 10		0 1 ml 10 41 × 10³		0 1 ml 10 1 41 × 10⁴	
days	TR	days	TR	days		TR	days	TR	days	TR	days	TR	days	TR	days	TR	days
223	3	82	0	86	Dubos Chicago1	0	90	8	88	5	87	15	90	16	93	19	92
244	0	87	5	92		2	102	8	93	13	91	14	100	17	101	19	101
244	3	88	3	93		2	104	11	95	3	92	15	100	19	105	21	108
244	0	91	0	95		5	104	9	97	14	97	16	105	16	105	18	108
44	0	91	5	97		0	104	0	99	12	99	15	108	19	106	18	109
244	0	94	0	99		0	107	0	100	14	99	18	108	19	109	20	119
244	0	95	5	100		4	107	13	105	12	104	16	109	17	116	20	125
244	3	96	6	102		0	108	9	107	9	104	14	120	17	120	20	128
44	0	96	0	105		0	109	13	110	16	105	17	123	15	120	21	132
244	0	97	0	107		7	109	12	118	14	105	14	128	17	125	16	137
44	2	100	5	108		0	111	14	130	13	107	18	131	18	127	19	140
244	0	103	0	109		12	118	5	131	14	107	16	134	15	128	19	145
244	0	107	7	114		7	118	7	132	18	109	11	138	15	131	19	146
44		07	0	117		0	119	2	135	13	112	15	141	19	131	15	147
244	0	8	3	125		0	119	12	138	9	126	16	144	17	135	21	148
44	0	1 4	3	1 9		6	131	13	141	15	127	14	146	16	136	18	148
244	0	130	2	131		0	133	14	144	10	127	16	151	18	142	19	152
244	3	113	0	13		13	140	7	151	13	131	15	153	20	148	17	157
244	0	160	0	135		0	147	11	173	13	139	12	154	18	162	20	184
244	0	173	7	168		9	164	10	189	12	158	16	160	14	185	19	187

= killed

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Statens Seruminstitut Pneumococcus Department, Copenhagen Denmark

RESISTENCE OF PNEUMOCOCCI IN PATHOLOGICAL SPECIMENS

By

ERNA LUND

Received 13 iii 67

Pneumococci can be identified by a Neufeld capsule reaction long after the organism has died Specimens of sputa spinal fluids and pus all containing *pneumococci were kept in a refrigerator at 4° C for about* 18 months and examined at intervals for growth and capsule reaction Diagnostic Pneumococcus sera *produced* at Statens Seruminstitut (*Lund* 1963 1966) was used for the Neufeld tests

The material consisted of 20 sputa 13 spinal fluids and 3 pus all received from the Diagnostic Department Statens Seruminstitut Cultures on 10 per cent blood agar and capsule reaction tests were made daily on all specimens for the first 2 weeks twice a week for 2 months and once weekly thereafter As long as any pneumococci were observed the reaction was recorded as positive

The *pneumococci remained* alive and gave a capsule reaction for longer periods in sputa than in spinal fluids and pus Table 1 shows that Pneumococcus type 3 in two sputa was found alive for a longer period than that in which it was possible to observe the capsule in a Neufeld test

In one of the sputa it was impossible to isolate pneumococci because of a heavy growth of proteus but pneumococcus type 7 was identified by Neufeld tests for 21 days

Whilst type 3 gave a capsule reaction for relatively few days in some specimens types 7 11 and 19 reacted for more than 500 days

The pneumococcal types 3 19 and 37 have bigger capsules than the other types and these big capsules are usually less stable than the smaller ones It has been found (*Holm & Mørch* 1945) that vaccines of types with big capsules do not keep as long as other pneumococcal vaccines so it was surprising to observe a capsule reaction with a type 19 in one of these sputa for 515 days as this type 19 had rather large capsules

The minimum survival time of pneumococci in sputa was 5 days and the maximum 135 days The shortest period for a positive Neufeld reaction was 8 and the longest 575 days The average for living cells was 35 days and for capsule reaction 145

TABLE 1

Pneumococci in 20 Sputa at 4 C

Pn-type	duration in days of growth on bloodagar	duration in days of capsule reaction
3	5	12
3	19	8
3	20	9
6	5	16
6	27	56
7	overgrown by proteus	21
7	10	515
9	19	105
9	56	63
10	135	189
11	34	63
11	54	575
16	14	224
17	40	161
19	12	515
22	25	35
23	9	42
33	42	196
34	10	12
34	42	91
average	35	145

TABLE 2

Pneumococci in 13 Spinal Fluids and 3 Pus at 4 C

	Pn-type	duration in days of growth on bloodagar	duration in days of capsule reaction
spinal fluids	3	2	5
	3	19	13
	4	12	45
	6	9	463
	10	14	147
	10	28	35
	14	27	160
	14	32	46
	18	21	35
	19	11	42
	19		7
	19		49
	23		11
pus	23	2	63
	34		210
	28		7
	average	16	85

The corresponding results for pneumococci in spinal fluids and pus are given in Table 2 Two specimens of pus were from sinus maxillaris (pn 23 and pn 34) and one from a pleural cavity (pn 28)

In two of the specimens of spinal fluid the pneumococci—a type 3 and a type 19—survived for periods longer than that in which they gave capsule reactions

The minimum survival time in spinal fluids and pus was only two days and the maximum 32 The shortest period for a positive capsule reaction was here 5 days and the longest 463 The average for living cells was 16 and for capsule reaction 85 days

SUMMARY

On an average pneumococci may survive at 4 C for 35 days in sputum and for 16 in spinal fluids and pus They may be identified by capsule reaction for 145 days in sputum and 85 in spinal fluids and pus

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Leo Pharmaceutical Produts Ballerup and the University Institute of Microbiology
Copenhagen

FUSIDIC ACID AND DERIVATIVES

Inhibition of Protein Synthesis in Cell Free Systems from Escherichia coli *and* Bacillus stearothermophilus

By

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Fusidic acid (Fig. 1) an antibiotic useful in the treatment of staphylococcus infections in man (*Barber & Garrod* (1)) has recently been shown to inhibit protein synthesis in intact cells of sensitive Gram positive bacteria as well as in spheroplasts and cell free systems from the naturally resistent Gram negative *E. coli* (*Yamaki* (2) and *Harvey, Knight & Sih* (3))

The mode of action of the inhibitor was further investigated (*Harvey, Knight & Sih* (3)) using *E. coli* cell free extracts. It was reported that fusidic acid did not inhibit the activation or transfer of phenylalanine to tRNA. The formation of the ternary complex of ribosomes mRNA

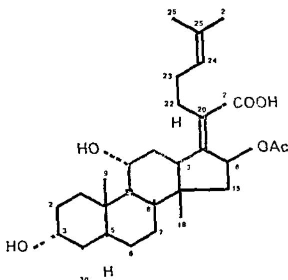


Fig. 1
Fusidic Acid

and amino acyl tRNA appeared to be rather unaffected although the heavy polysome fraction seems considerably reduced It was concluded that the antibiotic inhibits one of the later steps in protein synthesis

Based upon the observation that protein synthesis was inhibited in spheroplasts and cell free systems of *E coli* it was postulated that the lack of activity of fusidic acid against this organism is due to impermeability of the *E coli* cell wall

The inhibitory activity of a series of fusidic acid derivatives against a number of Gram positive and Gram negative bacteria has been reported (*Godtfredsen von Daehne & Tybring* (4)) All of the derivatives tested with the exception of 24 25 dihydrofusidic acid showed a lower activity than that of fusidic acid To investigate if the low activity *in vivo* of the derivatives was due to a decreased permeability a number of these were tested *in vitro* for inhibition of protein synthesis in cell free systems of *E coli* and *B stearothermophilus* and the results found compared with their antibacterial activity against a sensitive strain of *Staphylococcus aureus*

MATERIALS AND METHODS

In the experiments reported below crude cell free extracts were used for protein synthesis *in vitro* The system from *E coli* was prepared as described by *Forchhammer* & *Kjeldgaard* (6) measuring the incorporation of amino acids into polypeptides coded for by the natural messenger present in RNA extracted from *E coli* cells The system from *B stearothermophilus* NCA 2184[1] was prepared by the method of Fried man and Weinstein (7) using the synthetic messenger poly U to stimulate protein synthesis The (^{14}C) amino acids incorporated in polypeptides at 37 *in vitro* were precipitated with 2 volumes of 10 per cent trichloro acetic acid (TCA) heated to 96 for 20 minutes and prepared for counting in a gas flow counter (Frieseke Hoepfner F H 411 FH 49)

A fusidic acid resistant strain of *B stearothermophilus* was developed through passages in increasing concentrations of the compound The IC_{50} increased from 0 1 µg/ml to 10 µg/ml

Chemicals and reagents were supplied from the firms listed in Ref 3 and 6

RESULTS

Differences in ability to penetrate the cell wall might account for the varying *in vivo* activity of fusidic acid and some of its derivatives To evaluate this effect measurements of protein synthesis *in vitro* should be compared with the *in vivo* activity Two *in vitro* protein forming systems were used one isolated from a naturally resistant *E coli* strain and one from a sensitive strain *B stearothermophilus* The results obtained in these systems of the inhibitory action of fusidic acid and various derivatives are presented in Table 1 The last column shows the *in vivo* activity against a sensitive *Staphylococcus aureus* strain I Ref 4

The protein forming systems show the same response to the 22 synthetic derivatives with a few minor differences Only compounds 1 4

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[1] Kindly supplied by Professor C J Sih University of Wisconsin

TABLE 1

Protein Synthesis in Cell Free Systems

Compounds listed in Fig 2	Concentration µg/ml	*E coli*	B stearotherm	*Staph aureus* IC50 µg/ml
Control	0	100	100	
1	1	81		
1	10	45	40	
1	100	24	20	0 058
2	100	65	72	6 3
3	100	92–110	75	25
4	100	54	52	0 32
4	100	70	74	4 0
6	100	75	68	25
7	100	82	93	20
8	100	81	73	13
9	100	49	36	0 22
10	100	23	42	0 071
11	10	102	93	79
12	10	95–100	99	16
13	10	114	95	71
14	100	91	98	250
15	100	93	91	5 0
16	10	83	95	40
17	10	90–93	103	124
18	10	84	87	4 5
19	100	85	85	10
20	100	80	94	3 0
21	100	98	99	5 0
22	10	10	100	32
23	100	99	103	310

Protein synthesis is measured as an increase in hot TCA precipitable [14C] amino acid during incubation at 37 C This is in the control approx 1500 cpm with [14C] leucine (spec activity 150 mC/mM diluted 20 fold with [12 C] leucine) and approx 400 cpm with [14C] phenylalanine (spec activity 292 mC/mM diluted 20 fold with [12 C] phenylalanine) The values are means of duplicate assays

Compounds numbers 11 12 13 16 17 18 and 22 are insoluble at 100 µg/ml under assay conditions and therefore measured at the lower concentration

TABLE 2

Protein Synthesis in Cell Free Systems

Fusidic acid µg/ml	Sensitive str[illegible] *B stearoth[illegible]* (IC50 – [illegible])	Resistant strain of *B stearothermophilus* (IC50 = 10 µg)
Control		100
1	[illegible]	90–107
10	[illegible]	90–110
100	[illegible]	95

can be established either by a decrease in permeability of the cell wall or in sensitivity of the protein forming machinery to the antibiotic

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COMPARISON OF METHODS FOR ESTIMATION OF ANAEROBIC PRODUCTION OF ACID FROM GLUCOSE AND MANNITOL IN STAPHYLOCOCCI AND MICROCOCCI

By

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Received 13 iii 67

The International Subcommittee on Taxonomy of Staphylococci and Micrococci proposed to separate staphylococci from micrococci by use of the ability of staphylococci to grow and produce acid from glucose when incubated under anaerobic conditions (1965a) The Subcommittee recommended (1965b) a standard method in view of the demonstration by *Cowan & Steel* (1964) of the variety of results obtainable using methods proposed by different workers

The object of the present study was to compare results of the proposed standard method with other methods for the estimation of anaerobic production of acid from glucose and mannitol The other methods were (i) final pH in the *Evans* medium (1955) (ii) *Baird Parker s* medium (1963) (iii) *Hugh & Leifson s* medium (1953) and (iv) *Mossel & Martin s* medium (1961)

MATERIAL AND METHODS

Strains 29 strains of gram positive and catalase positive cocci were received from the National Collection of Type Cultures (NCTC) London and from the Czechoslovak Collection of Microorganisms (CCM) Brno The names and numbers under which they were received as well as their DNA ba e mpos on expressed as moles per ent f guanine + cytosine (GC per cent) [illegible] ted n Table 1 B ochemical characteristics of the strains were publi [illegible] n a p e us paper (*Mortensen & Kocur* 1967)

Evans medium Trypticase (BBL) 1 0 per cent yeast extract (Difco) 0 5 per cent NaCl 0 5 per cent K_2HPO_4 0 1 per cent carbohydrate 1 0 per cent (*Evans et al* 1955) Dispensed in 5 ml amounts in 150 × 13 mm test tube inoculated and incubated at 35 C in an anaerobic jar pH readings were performed on the second fourth and sixth day of incubation (for details see *Mortensen & Kocur* 1967)

Baird Parker s medium $NH_4H_2PO_4$ 0 1 per cent KCl 0 02 per cent $MgSO_4$ ($7H_2O$) 0 02 per cent yeast extract 0 1 per cent bromocresol purple 0 004 per cent carbohydrate 1 0 per cent agar 0 2 per cent pH 7 0 (*Baird Parker* 1963)

Hugh & Leifson s medium Peptone (Orthana special) 0 2 per cent NaCl 0 5 per cent K_2HPO_4 0 03 per cent agar 0 3 per cent bromthymol blue 0 003 per cent carbohydrate 1 0 per cent pH 7 1 (*Hugh & Leifson* 1953)

registered in the remaining strains of section B or in any strains of section C in any of the tubed media used in this study

TABLE 2

Production of Acid from Mannitol under Anaerobic Conditions of 10 Staphylococcus Strains in Five Different Media after Four Days Incubation at 35 C

Strains	Coll number	Average pH value in Evans medium	Baird Parker s medium	Hugh & Leifson s medium	Mossel & Martin s medium	Standard medium	Comm[illegible]
S aureus	CCM 529	4 9		+	+	+	-
S epidermidis	CCM 901	6 8	—	—	—	—	-
S lactis	NCTC 7944	4 7		+	+	+	-
S aureus	NCTC 4136	4 9		+	+	+	+
S aureus	NCTC 8532	4 8		+	+	+	+
S aureus	NCTC 6571	4 9	+	+	+	+	+
S aureus	NCTC 4163	5 3		+			+
S saprophyticus	NCTC 7292	6 6	or —	+ or —	-	or —	-
S saprophyticus	NCTC 7612	6 4		+ or —	—	or —	-
S lactis	NCTC 189	6 9	or —	+	—	—	-

+ = Colour change throughout the tube within four days
— = *No colour change*
= Colour change in the upper part of the tube
= For details see *Mortensen & Kocur (1967)*

By the criterion set up and method recommended by *Mossel* (1962) 2 of the coagulase negative strains and 3 of the coagulase positive strains in section A were fermenters of mannitol and these were positive in the Standard medium as well Hugh & Leifson s medium was a more sensitive means to demonstrate production of acid from mannitol while Baird Parker s medium proved less sensitive having only one positive strain in section A Three strains of section B produced variable and minor amounts of acid in Baird Parker s and the Subcommittee s media and were irregularly positive in Hugh & Leifson s medium

In Evans medium the pH values ranged from 4 7 to 4 9 for strains which were positive in Mossel & Martin s medium and the Standard medium and from 5 3 to 6 9 for strains which did not change the colour of the medium throughout the tube

DISCUSSION

Cowan & Steel (1964) compared different methods by which to estimate the production of acid from glucose in staphylococci and micrococci They concluded that the best agreement was attained when anaerobic fermentation of glucose was tested by Baird Parker s method by peptone water cultures in an anaerobic jar and by the method suggested by *Evans et al* when a final pH of less than 5 0 was required

Our data confirm the agreement of results by Baird Parker s method

and Evans method when a final pH of less than 5 0 is required and show agreement of these two tests with the Standard method proposed by the Subcommittee on the Taxonomy of Staphylococci and Micrococci (1965b) and Mossel & Martin s medium (1961)

The lack of conformity between Hugh & Leifson s test and the other tests may be due to several factors The depth of this medium is only 1 5 inches (a little less than 4 cm) against the more than 3 inches (8 cm) in the other semi solid media Since a number of the strains positive in Hugh & Leifson s test changed the colour of the medium in the upper half of the other media this difference in depth of the medium may be important Furthermore the increase in hydrogen ion concentration which will change the colour of the indicator is smaller for bromthymol blue the indicator used in Hugh & Leifson s test than for bromocresol purple the indicator used in the other semi solid media This is due to the lower pK_{In} of bromocresol purple

The results also confirm the existence of a group of weak producers of acid from glucose as previously reported by *Cowan & Steel* (1964) who found 15 of their 87 strains reached a final pH between 5 0 and 6 0 in Evans medium *Mortensen & Kocur* (1967) found this intermediate group included strains of both high and low GC per cent and *Auletta & Kennedy* (1966) found weak producers of acid from glucose among strains with a low GC per cent

These results show that the monothetic division of aerobic gram positive catalase positive cocci based on the anaerobic production of acid from glucose is not completely satisfactory The strong producers of acid are staphylococci be several criteria but some of the weak or variable producers of acid from glucose are also candidates for the staphylococcus group

The variability of the production of acid in strains of the intermediate group will occasionally cause strains with a high GC per cent to be identified as staphylococci and strains with a low GC per cent as micrococci A strict adherence to the proposal of the Subcommittee thus entails a certain number of misplacements although the recommendation of the Subcommittee probably is the best possible for the time being

The results of the production of acid from mannitol confirm previous investigations of this quality

SUMMARY AND CONCLUSIONS

Twenty nine strains of catalase positive cocci were examined for acid production from glucose and mannitol under anaerobic conditions by five different tests These were electrometric determination of pH in Evans medium and observation of colour changes in four semi solid media with indicators (Baird Parker s Hugh & Leifson s Mossel & Martin s media and the Standard medium proposed by the Subcommittee on the Taxonomy of Staphylococci and Micrococci)

The results of tests in Mossel & Martin's, Baird-Parker's and the Standard media with glucose correlated well with pH determinations in Evans medium: all strains lowering pH below 5.0 were positive in these semi-solid media. With the exception of a few strains which more slowly produce varying and minor amounts of acid (*Mortensen & Kocur 1967*) and which irregularly give positive results in Baird-Parker's and the Standard media, all other strains were negative.

Hugh & Leifson's medium proved more sensitive to minor amounts of acid than the other test media.

For routine work with tubed indicator media, use of Mossel & Martin's, Baird-Parker's and the Standard media will serve to distinguish strong producers of acid from glucose among the gram-positive, catalase-positive cocci.

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PROPERTIES OF ANAEROBIC GRAM POSITIVE RODS CAPABLE OF 7α DEHYDROXYLATING BILE ACIDS

By

TORE MIDTVEDT

Received 18 ii 67

The two primary bile acids formed in man and most mammalian species cholic acid and chenodeoxycholic acid are excreted in the bile conjugated with taurine or glycine In the intestinal tract they are attacked by microbial enzymes and transformed into various metabolites The predominant type of transformation involves the elimination of the 7α hydroxyl group leading to the formation of deoxycholic acid and lithocholic acid respectively (12)

The isolation of faecal microorganisms capable of this 7α dehydroxylation *in vitro* has been reported elsewhere (12) In all eight strains with this capacity were isolated seven from rat faeces and one from human faeces The following investigation describes some of the properties found in these eight strains with a trial for their identification

MATERIALS AND METHODS

Bacterial Strains

The seven strains from rat faeces were designated I II III V VI VII and VIII and the human strain was labelled KD The bacterial strains used for comparison are listed in Table 1

General Bacteriological Procedures

As all the strains grew well in Todd Hewitt broth (Oxoid) this was used as the main stock culture medium (TH broth) The strains were subcultured once or twice weekly In addition the strains were gr[illegible]n [illegible] red in Brain Liver Heart medium (Difco) and Fluid Thioglycollate medium (Difco) (BLH medium and FT medium) More than 100 transfers were made N[illegible] in the morphological or biochemical properties of the strains were [illegible] nd ating ati f ctor stability of the strains

Unless otherwise stated the tub[illegible] e incubated anaerobically at 37 C using the pyrogallol method (12) after b[illegible] and cooling of the medium Cultivations on agar plates were made in [illegible] bi jar under an atmosphere of 95 per cent hydrogen and 5 per cent CO

Surface colonies were studied in 1.5 per cent agar plants of TH BLH and FT medium Loeffler serum slant and horse blood agar plates Formation of spo[illegible]s

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This investigation [illegible] supported by [illegible] rel grants from the National Institutes of Health Public Health Service (A-1933) the Swedish Medical Research Council (90 and 06) and the Wallenberg Foundation Stockholm Sweden

Present address K[illegible] in W Wilhelmsen og Frue Bakteriologiske Institutt Oslo Norway

The results of tests in Mossel & Martin s Baird Parker s and the Standard media with glucose correlated well with pH determinations in Evans medium all strains lowering pH below 5 0 were positive in these semi solid media With the exception of a few strains which more slowly produce varying and minor amounts of acid (*Mortensen & Kocur 1967*) and which irregularly gave positive results in Baird Parker s and the Standard media all other strains were negative

Hugh & Leifson s medium proved more sensitive to minor amounts of acid than the other test media

For routine work with tubed indicator media use of Mossel & Martin s Baird Parker s and the Standard media will serve to distinguish strong producers of acid from glucose among the gram positive catalase positive cocci

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was also studied after 1–60 days at 30 C and 37 C in Cooked Meat medium (Difco) Egg Meat medium (Difco) a casein peptone medium (?) and it TH broth and BLH medium with addition of 0 003 per cent thioglycollic acid and 0 2 per cent starch (18)

When transferring the strains smears were always made and Gram stained A modified Ziehl Neelsen technique (5) and the technique described by *Ashby* (1) was used for demonstration of spores Demonstration of volutin granula was made according to *Layburn* (14) and capsules according to *Duguid* (8)

Physiological Properties

Motility was studied by phase contrast microscopy of cultures in TH broth BLH medium and FT medium after 3 6 12 24 and 48 hours of incubation It was also studied in semi solid media prepared by adding 0 5 per cent agar (28) to TH broth BLH medium FT medium Thioglycollate medium without Dextrose or Indicator (Difco) (TDI medium) and in Motility Sulphide medium (Difco) (MS medium) after incubation at 37 C and at room temperature

Growth analyses were performed in Pyrex tubes (Coleman Instruments Inc Marywood Ill USA) The tubes were filled with 12 ml of medium and shaken during incubation Optical densities (O D) were determined in a Coleman Junior Spectrophotometer at 525 mμ Growth rates at different temperatures (45 C 37 C, 30 C and room temperature) were tested in the strains II VII and KD TDI medium was employed with and without the addition of 1 per cent d galactose

Sensitivity to heat was tested in the strains II VII and KD After cultivations in TH broth BLH medium and FT medium for 3 days and 8 days at 37 C aliquots of 0 5 ml were transferred to 9 5 ml of TH broth BLH medium and FT medium and heated to the temperature selected (60 C 70 C 80 C 90 C) for 1 min 5 min or 20 min Tubes containing the test media were used as controls The temperatures were controlled with a reading thermometer within the limits ± 0 5 C The tubes were then incubated anaerobically at 37 C and checked for growth up to 14 days

Susceptibility to air was tested in all strains After incubation in TH broth for 3 days and 8 days aliquots of 5 ml were transferred to sterile tubes containing a glass rod 2–3 cm long and shaken aerobically at 37 C Aliquots of 0 5 ml were taken at intervals and inoculated into TH broth and FT medium Growth was recorded after 7 days

Growth Requirements

Standard media—One per cent solutions (w/v) of Tryptone Tryptose Neopeptone Casamino acids Beef extract and Yeast extract (Difco) with and without addition of a solution of mineral salts in phosphate buffer (15) were inoculated with 0 1 ml of a 72 hour old culture in TH broth and incubated up to 12 days In addition growth was tested in Tryptone Soya broth (Oxoid) Trypticase Soy broth (BBL) and Penassay broth (Difco)

Synthetic media—Growth was tested in Glutamic Assay medium (Difco) with and without 40 mcg per ml of l glutamic acid and 0 5 mcg per ml of folic acid Inoculum for assays was prepared by subculturing the strains in Micro Inoculum broth (Difco) for 72 hours aliquots of 0 1 ml were used to inoculate 12 ml of the test medium

Vitamin requirements—Folic Acid Assay medium Niacin Assay medium and Riboflavin Assay medium (Difco) were prepared (7) and inoculated as above Vitamin dependent strains (Table 1) were used as controls

Biochemical Properties

Fermentation of carbohydrates—TDI was chosen as basic medium for fermentation studies and the carbohydrates were added as sterile filtered solutions in a final concentration of 1 per cent (w/v) Aliquots of 0 1 ml of 72 hour old cultures in TDI medium were used for inoculation The pH was determined after 1 2 3 6 and 12 days of incubation using a pH 22 pH meter from Radiometer Copenhagen Denmark The determinations were made immediately after the removal of the anaerobic seals

Other biochemical tests—Catalase and oxidase tests were performed by pouring 3 per cent or 10 per cent peroxide and 1 per cent aqueous solution of dimethyl p phenyl diaminohydrochloride respectively on surface colonies Formation of indol and skatol was tested after 3 6 and 12 days of incubation in the medium described

by *Spray* (26) and in TH broth BLH medium and TDI medium (with/without 1 per cent dextrose) Production of indol was determined with Ehrlich s reagent and skatol was determined using the vanilin test (20) Reduction of nitrate to nitrite was investigated according to *Ellen* (3) after 3 6 and 12 days of incubation in the medium described by *Spray* (26) and in TDI medium (with/without 1 per cent dextrose) with addition of 0 1 per cent KNO_3

Liquefaction of gelatin was tested in TH broth FT medium and TDI medium with 7 5 per cent or 15 per cent of gelatin (Oxoid) added and in MS medium The tubes were incubated at 37 C and at room temperature up to 60 days Production of hydrogen sulphide from l cystine was tested in MS medium incubated at 37 C and at room temperature

Voges Proskauer reaction was carried out according to *Barritt* (2) after incubation for 8 4 6 and 12 days in TDI medium containing 1 per cent dextrose Growth in milk media was tested in Litmus Milk medium (Difco) and Crookes Mill medium (Oxoid) Incubations were continued up to 28 days The proteolytic properties of the strains were also tested in Cooked Meat medium Egg Meat medium and on Loeffler s serum slants

Antibiotic Sensitivity

The sensitivity of the strains to the antibacterial drugs listed in Table 8 was determined by serial dilutions of the drugs in Penassay broth and Tryptone Soya broth The tests were made in two fold drug dilutions steps and the inoculum was 0 2 ml of a 72 hour old culture in TH broth into 10 ml Minimum inhibitory concentration (M I C) was determined after 72 hours of incubation

Pathogenicity to Mice

After cultivations in TH broth and FT medium for 3 days and 8 days aliquots of 0 5 ml or 1 ml were injected intraperitoneally into mice of the NMRI strain 60–90 days old The mice were observed for 10–30 days

RESULTS

Morphology

Surface growth on solid media —No growth was obtained under aerobic conditions Cultivated anaerobically on TH or BLH agar slants all strains showed minute transparent colonies after 36–42 hours of incubation After 3–4 days the diameter of the colonies was 1–3 mm they were circular with an entire edge smooth and glistening surface and greyish white colour During the first 3–4 days of incubation the consistency of the colonies was friable and then became more butyrous *Emulsified in TH broth they formed homogenous suspensions* On FT agar slants the colonies varied more widely in size than on TH and BLH agar slants Haemolytic zones were never seen on horse blood agar plates and no liquefaction of the media occurred on Loeffler s serum slants

Growth in fluid media —No growth was obtained under aerobic conditions in TH broth When aliquots of 0 2 ml from 72 hour old cultures were used to inoculate 12 ml TH broth growth under anaerobic conditions started after a lag phase lasting at most 12 hours Highest values of densities were observed after 36–60 hours of incubation When the strains were cultivated in BLH medium or FT medium without any anaerobic seals they usually failed to grow However in the lower part of some of the tubes scanty growth gradually developed

Anaerobic incubation in these two media gave good growth after about the same lag phase as that in TH broth

Microscopic examination —The microscopic appearance of the strains was virtually identical Gram staining were taken every day from cultures on TH agar slants The first 3 samples showed slender 0 2–0 8 × 2–8 μ Gram positive rods with straight axis In older cultures many curved forms occured and both Gram positive and Gram negative rods were observed The microscopic appearance of the strains in TH broth BLH medium and on BLH agar slants was the same as that on TH agar slants Cultivations in FT medium and on FT agar slants showed a tendency towards pleomorphism with terminal swelling and central thickening These forms were always Gram positive and could not be stained with spore stains After short reincubation in fresh media the pleomorphic forms disappeared Despite pleomorphism no true bifid forms were observed

Spores volutin granula and capsules were never demonstrated

Physiology

Motile bacteria were not demonstrated by microscopy or in the semi solid media *Clostridium butyricum Cl tetani Cl perfringens* and *Lactobacillus bifidus* were used as controls (Table 1)

None of the strains grew at 45 C whereas all grew at 37 C 30 C and at room temperature Table 2 shows that the most rapid growth occurred at 37 C indicating that the strains are mesophilic

TABLE 2

Growth Rates at Varying Temperatures of the Strains II VII and KD in TDI Medium with Addition of 1 Per Cent of Calalase

Temperature	Strain	0	12	24	36	48	72	96
		Hours of incubation						
37 C	II	0	0	0 24	[illegible]	[illegible]	[illegible]	0 60
	VII	0	0	0 10	0 1[illegible]	[illegible]	0 [illegible]0	0 20
	KD	0	0 19	0 4[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
30 C	II	0	0	[illegible]	[illegible]	[illegible]	[illegible]	0 64
	VII	0	0	[illegible]	0 10	[illegible]	[illegible]	0 33
	KD	0	0	[illegible]	[illegible]	[illegible]	0 44	0 38
20 C	II	0	0	0	0	[illegible]	0 44	0 64
	VII	0	0	0	0	0 09	0 [illegible]1	0 3[illegible]
	KD	0	0	0 08	[illegible]	0 5[illegible]	[illegible]	0 59

These samples were analysed up to 156 hours of incubation but the highest values were reached within 96 hours

§ Room temperature approximately 20 C

The results are given in terms of optical density Each figure represents the mean value of two different samples [illegible] from the mean within the limit ± 0 0[illegible]

Values ≦ 0 05 are [illegible] as 0

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		0	12	24	36	48	72	96*
37 C	II	0	0	0 24	0 [illegible]	0 [illegible]	0 [illegible]	0 66
	VII	0	0	0 18	0 [illegible]	0 23	0 70	0 70
	KD	0	0 19	0 49	0 [illegible]	0 23	0 27	0 [illegible]
30 C	II	0	0	0 09	0 [illegible]	0 50	0 [illegible]	0 64
	VII	0	0	0	0 10	0 22	0 3[illegible]	0 33
	KD	0	0	0 [illegible]	0	0 [illegible]	0 44	0 35
20 C§	II	0	0	0	0	0 09	0 44	0 61
	VII	0	0	0	0	0 09	0 2[illegible]	0 3
	KD	0	0	0 09	0	0 5	0 50	0 59

* The samples were [illegible] up to 156 hours of incubation but the highest values were reached with[illegible] 96 hours
§ Room temperature approximately 20 C
The results are given in terms of [illegible] Each figure represents the [illegible] value of two different [illegible] the mean within the limit [illegible]
Values [illegible] 0 0 [illegible]

None of the strains survived 80 C for 5 minutes or 90 C for 1 minute. The results in Table 3 indicate that the strains are moderately thermoresistant but the resistance is not as high as it usually is for spore forming bacteria.

TABLE 3

Growth after Exposure to Heat in the Strains II VII and KD Incubated for 3 and 8 Days before the Heating Procedures

Test temperature	Duration	Growth in no of tubes/ no of tube tested					
		3 day old cultures			8 days old cultures		
		II	VII	KD	II	VII	KD
60 C	1 min	10/12	12/12	10/12	7/12	11/12	9/12
	5 min	6/12	6/12	4/12	2/12	4/12	1/12
	20 min	2/12	3/12	3/12	3/12	2/12	1/12
70 C	1 min	10/24	12/24	3/24	4/24	2/24	0/24
	5 min	9/24	7/24	1/24	6/24	1/24	0/24
	20 min	3/24	5/24	1/24	4/24	2/24	0/24
80 C	1 min	1/12	4/12	1/12	1/12	0/12	0/12

All the strains survived 2 hours of aerobic incubation in FH broth but not 10 hours under the same conditions. The results indicate that our strains are susceptible to exposure to air.

Growth requirements—None of the strains grew in the Tryptone Tryptose Neopeptone Casamino acids Beef extract and Yeast extract media. In Tryptone Soya broth Trypticase Soy broth and Penassay broth visible growth was obtained after about 24 hours of incubation.

None of the strains grew in the Glutamic Assay medium with or without addition of 1 glutamic acid (Table 4). On addition of folic acid all the strains except strain KD were able to grow. Addition of glutamic acid did not significantly affect growth.

TABLE 4

Growth of the Eight Strains after 72 Hours of Incubation in Glutamic Assay Medium with/without Addition of l Glutamic Acid and Folic Acid

l glutamic acid in mcg/ml	Folic acid in mcg/ml	Strain							
		I	II	III	V	VI	VII	VIII	KD
0	0.02	0	0	0	0	0	0	0	0
40	0.02	0	0	0	0	0	0	0	0
40	0.5	0.08	0.12	0.18	0.13	0.18	0.20	0.06	0
0	0.5	0.08	0.09	0.20	0.12	0.20	0.24	0.08	0

According to the manufacturers description.
The results are given in terms of optical density. *Each figure represents the mean value of two different samples deviation from the mean within the limit* ± 0.05.
Values ≦ 0.05 are quoted as 0.

TABLE 5

Growth of Strain II and S faecalis ATCC 8043 after 72 Hours of Incubation in Folic Acid Assay Medium Containing Varying Concentrations of Folic Acid

Strain	Growth in media containing folic acid in concentrations of (mcg per ml)					
	0	0 0005	0 005	0 05	0 5	5
Strain II	0	0	0	0 24	0 33	0 44
S faecalis	0	0 21	0 48	0 45	0 44	0 52

The results are given in terms of optical density Each figure represents the mean value of two different samples deviation from the mean within the limit ± 0 05 Values ≦ 0 05 are quoted as 0

The dependence of strain II on folic acid was more thoroughly tested in Folic Acid Assay medium Optimal growth was obtained at the highest concentration of folic acid (*i e* 5 mcg per ml) (Table 5) With concentration of folic acid lower than 0 05 mcg per ml the growth was almost unmeasurable

TABLE 6

Growth of the Isolated Strains after 72 Hours of Incubation in Vitamin Test Media with/without the Addition of Vitamins

Medium	Vitamin added mcg/ml	Folic acid added mcg/ml	Strain I	II	III	V	VI	VII	VIII	KD	Control
Folic Acid Assay medium		0	0	0	0	0	0	0	0	0	0
		0 0005	0	0	0	0	0	0	0	0	0 16
		0 5	0 35	0 38	0 41	0 20	0 25	0 49	0 24	0 11	0 46
Niacin Assay medium	0	0	0	0	0	0	0	0	0	0	0 09
	5 0	0	0	0	0	0	0	0	0	0	0 83
	5 0	0 5	0 49	0 31	0 32	0 51	0 64	0 50	0 45	0 21	0 83
	0	0 5	0 51	0 26	0 25	0 59	0 64	0 45	0 46	0 16	0 10
Riboflavin Assay medium	0	0	0	0	0	0	0	0	0	0	0
	5 0	0	0	0	0	0	0	0	0	0	0 83
	5 0	0 5	0 08	0 13	0 30	0 10	0 11	0 26	0 29	0	0 87
	0	0 5	0	0	0	0	0 14	0 07	0	0	0 10

cfr Table 1

The results are given in terms of optical densities Each figure represents the mean value of two different samples deviation from the mean within the limit ± 0 0 Values ≦ 0 05 are quoted as 0

From Table 6 it is apparent that all the strains required folic acid None of them seemed to require niacin When cultivated in Riboflavin Assay medium five of the strains grew rather scanty even after addition of large amounts of both folic acid and riboflavin However in the remaining three strains (strain III VII and VIII) rather good growth was obtained in the medium containing folic acid and riboflavin where

as growth in the medium containing folic acid was scanty. Thus strains III, VII and VIII seemed to be dependent upon riboflavin.

TABLE 7

Fermentation of Carbohydrates in Three of the Isolated Strains after 3 Days at 37° C

Substance	Strain II			Strain VII			Strain KD			Controls		
	pH	gas	O.D.	pH	gas	O.D.	pH	gas§	O.D.†	pH	gas	O.D.
l arabinose	5.25	+++	0.65	5.60	+++	0.32	6.40	—	0.10	6.85	—	0
d xylose	5.20	+++	0.67	5.50	+++	0.38	5.45	+++	0.42	6.85	—	0
d fructose	5.35	+++	0.64	5.65	+++	0.53	5.35	+++	0.40	6.90	—	0
d galactose	5.35	++	0.68	5.55	++	0.44	5.60	+++	0.30	6.90	—	0
dextrose	5.80	++	0.44	5.60	+++	0.34	5.70	+++	0.32	6.80	—	0
maltose	6.65	—	0	6.60	—	0.06	5.25	++	0.76	7.00	—	0
saccharose	5.30	+++	0.81	5.45	+++	0.57	6.55	—	0.11	6.95	—	0
dulcitol	6.45	—	0.08	6.35	—	0.09	6.10	++	0.10b	6.95	—	0
d sorbitol	5.95	++	0.32	6.[illegible]	++	0.16	5.95	++	0.19	7.00	—	0
d mannitol	6.10	++	0.12	6.25	++	0.12	6.15	++	0.11c	7.00	—	0
Test medium without carbohydrate	6.65	—	0	6.60	—	0	6.50	—	0.08	6.95	—	0

Each figure represent the mean value of 2–20 different samples, deviation from the mean within the limit ± 0.20.

§ Gas filling up to 1/3 of the Durham tube indicated by +, up to 2/3 by ++, the whole tube by +++, no production of gas is indicated by —.

† Each figure represent the mean value of 4–10 different samples, deviation from the mean within the limit ± 0.05. Values lower than ≦ 0.05 are quoted as 0.

b O.D. 0.22 reached within 72 hours.

c O.D. 0.18 *reached within 72 hours.*

In addition the following substances were not fermented: d mannose, cellobiose, lactose, melibiose, trehalose, rhamnose, raffinose, melezitose, inulin, glycerol, adonitol, inositol and salicin. pH values were 6.45 or higher, no production of gas was observed and O.D. was ≦0.11.

Biochemistry

Fermentation of carbohydrates.—Strains II, VII and KD were selected and their fermentation capacity was tested. Production of acid and gas and change in optical density (O.D.) after 72 hours of incubation are given in Table 7. The strains fermented heterofermentatively (i.e. with production of both acid and gas) the following substances: d xylose, d galactose, dextrose, d fructose, d sorbitol and d mannitol. Moreover strain II and VII fermented l arabinose and saccharose, whereas strain KD fermented maltose and dulcitol. The results obtained after 6 and 12 days of incubation were virtually the same. The 5 strains not included in the table were found to act heterofermentatively upon d xylose, d galactose, dextrose and saccharose.

Other biochemical tests.—All the strains were catalase and oxidase negative. They did not produce indol or skatol, neither reduced nitrate to nitrite, liquefied gelatin nor produced acetylmethylcarbinol. In MS medium they produced hydrogen sulphide after 2–3 days at 37° C and

after 3–4 days at room temperature In milk media no production of acid or digestion of casein was observed Growth in Cooked Meat and Egg Meat medium was scanty and no digestion of the meat occurred All cultures of the strains were found to be odourless

Antibiotic Sensitivity

Except in two strains the results obtained in the two media used never differed more than one dilution step The results found in one of the media are listed in Table 8 The results indicate that our strains are sensitive or moderately sensitive towards the antibiotic tested (10 11) but are resistant towards nalidixic acid

TABLE 8

MIC of One Chemotherapeutic Agent and Some Antibiotics on the Growth of the Eight Strains in Tryptone Soya broth

Agent	Inhibitory range mcg/ml
Nalidixic acid	>100
Penicillin G	0 05–0 2
Ampicillin	0 2 –1 6
Oxytetracycline	<0 4 –1 6
Chloramphenicol	3 1 –6 3
Streptomycin	1 6 –6 3
Neomycin	<1 6 –6 3

Pathogenicity to Mice

All eight strains were injected intraperitoneally into 16 mice each None of them died during the observation period Post mortem examinations performed on some of the animals showed no signs of infections

DISCUSSION

Identification of the Eight Strains Capable of 7α Dehydroxylation — On the basis of the demonstrated morphological physiological and biochemical properties it may be concluded that the strains have most of their characteristics in common They are all anaerobic Gram positive rods However the classification and nomenclature in some of the species in this type of microorganisms is still an open question There are at present two systems of classification of anaerobic microorganisms the system of *Bergey's* Manual (4) and that of *Prevot* (21 23) As far as the nomenclature is concerned that of Bergey's Manual will be used in the following

Owing to the heterogeneity of the anaerobic Gram positive rods several genera have to be discussed in the identification of the eight strains The anaerobic Gram positive rods in question are the members of the genera *Actinomyces Corynebacterium Butyribacterium Clostridium*

is growth in the medium containing folic acid was scanty Thus strains III VII and VIII seemed to be dependent upon riboflavin

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	pH	gas	O D	pH	gas	O D	pH	gas§	O D †	pH	gas	O D
l arabinose	5 25	+++	0 65	5 60	+++	0 32	6 40	—	0 10	6 85	—	0
d xylose	5 90	+++	0 67	5 50	+++	0 38	5 45	+++	0 42	6 85	—	0
d fructose	5 35	+++	0 74	5 65	+++	0 53	5 3	+++	0 40	6 90	—	0
d galactose	5 35	++	0 68	5 55	++	0 44	5 60	+++	0 30	6 90	—	0
dextrose	5 80	++	0 44	5 60	+++	0 34	5 70	+++	0 32	6 80	—	0
maltose	6 65	—	0	6 60	—	0 06	5 25	++	0 16	7 00	—	0
saccharose	5 30	+++	0 81	5 45	+++	0 57	6 55	—	0 11	6 95	—	0
dulcitol	6 45	—	0 09	6 35	—	0 03	6 10	++	0 10b	6 95	—	0
d sorbitol	5 95	++	0 32	6 25	++	0 16	5 95	++	0 19	7 00	—	0
d mannitol	6 10	++	0 12	6 25	++	0 12	6 15	++	0 11c	7 00	—	0
Test medium without carbohydrate	6 65	—	0	6 60	—	0	6 50	—	0 08	6 95	—	0

Each figure represent the mean value of 2–20 different samples deviation from the mean within the limit ± 0 20

§ Gas filling up to 1/3 of the Durham tube indicated by + up to 2/3 by ++ the whole tube by +++ no production of gas is indicated by —

† Each figure represent the mean value of 4 10 different samples deviation from the mean within the limit ± 0 05 Values lower than ≦ 0 05 are quoted as 0

b O D 0 29 reached within 72 hours

c O D 0 18 reached within 72 hours

In addition the following substances were not fermented d mannose cellobiose lactose melibiose trehalose rhamnose raffinose melezitose inulin glycerol adonitol inositol and salicin pH values were 6 45 or higher no production of gas was observed and O D was ≦0 11

Biochemistry

Fermentation of carbohydrates—Strains II VII and K D were selected and their fermentation capacity was tested Production of acid and gas and change in optical density (O D) after 72 hours of incubation are given in Table 7 The strains fermented heterofermentatively (i e with production of both acid and gas) the following substances d xylose d galactose dextrose d fructose d sorbitol and d mannitol Moreover strain II and VII fermented l arabinose and saccharose whereas strain K D fermented maltose and dulcitol The results obtained after 6 and 12 days of incubation were virtually the same The 9 strains not included in the table were found to act heterofermentatively upon d xylose d galactose dextrose and saccharose

Other biochemical tests—All the strains were catalase and oxidase negative They did not produce indol or skatol neither reduced nitrate to nitrite liquefied gelatin nor produced acetylmethylcarbinol In MS medium they produced hydrogen sulphide after 2–3 days at 37 C and

after 3–4 days at room temperature In milk media no p od acid or digestion of casein was observed Growth in Cooked M a Eg, Meat medium was scanty and no digestion of the meat occ All cultures of the strains were found to be odourless

Antibiotic Sensitivity

Except in two strains the results obtained in the two media never differed more than one dilution step The results four of the media are listed in Table 8 The results indicate that are sensitive or moderately sensitive towards the antibiotic test 11) but are resistant towards nalidixic acid

TABLE 8

MIC of One Chemotherapeutic Agent and Some Antibiotics on the Eight Strains in Tryptone Soya broth

Agent	Inhibitory range mcg/ml
Nalidixic acid	>100
Penicillin C	0 05 0
Ampicillin	0 ? –1 6
Oxytetracycline	<0 4 –1 6
Chloramphenicol	3 1 –6 3
Streptomycin	1 (–6 3
Neomycin	<1 6 –6 3

Pathogenicity to Mice

All eight strains were injected intraperitoneally into 16 mice None of them died during the observation period Post mortem examinations performed on some of the animals showed no signs of lesions

DISCUSSION

Identification of the Eight Strains Capable of 7α Dehydroxylation
On the basis of the demonstrated morphological physiological and biochemical properties it may be concluded that the strains have most of their characteristics in common They are all anaerobic Gram rods However the classification and nomenclature in some species in this type of microorganisms is still an open question are at present two systems of classification of anaerobic organisms the system of Bergey s Manual (4) and that of Prevot As far as the nomenclature is concerned that of Bergey s Manual will be used in the following

Owing to the heterogeneity of the anaerobic Gram positive rods several genera have to be discussed in the identification of the eight strains The anaerobic Gram positive rods in question are the members of the genera *Actinomyces Corynebacterium Butyribacterium Clostridium*

and the tribe *Lactobacilleae* But in view of the morphological physio logical and biochemical properties of the strains investigated they can not be included in the genera *Actinomyces* or *Clostridium* However they have all some properties in common with species included in the genera *Corynebacterium* and *Butyribacterium* and in the tribe *Lacto bacilleae* In the following a comparison will be made between some of the properties of our strains and those described for these groups of organisms

Corynebacterium—This genus is divided into 33 species only 5 of which are stated to be anaerobic or microaerophilic (4) Three out of these 5 species are homofermentative and have to be excluded from this discussion thus leaving only 2 species *C avidum* and *C diphtero ides* *Corynebacterium avidum* is described as capable of coagulating and partly digesting milk liquefying gelatin fermenting trisaccharides as melezitose but incapable of fermenting xylose and arabinose thus differing distinctly from our strains in all these respects The descrip tion of *Corynebacterium diphteroides* in Bergey s Manual is rather scanty and gives the fermentation result of only dextrose *Prevot* (23) states that dextrose fructose galactose and maltose are usually fer mented The results of some other biochemical tests (*i e* gelatin lique faction milk coagulation nitrate reduction) for this species and for our strains are very similar However *C diphteroides* shows volutin gra nula (4 23) produces indol (4 23) is not thermoresistant (23) and may produce catalase (22 23) In view of these differences our strains cannot be included in this species

Butyribacterium—This genus has only one species *Butyribacterium rettgeri* (4) The morphological and biochemical properties in this species are very similar with those found in our strains However *B rettgeri* grows at 45 C (17 23) is anaerobic to microaerophilic (4 17) may liquefy gelatin (17) produce indol (23) ferment glycerol (23) and adonitol (17) These differences indicate that our strains do not belong to this species

Lactobacilleae—The last group to be discussed comprises the micro organisms included in the tribe *Lactobacilleae* This tribe is divided into 5 genera *Lactobacillus* *Eubacterium* *Catenabacterium* *Ramibac terium* and *Cillibacterium* (4) Owing to its motility the last genus will be excluded from the following discussion

The genera *Eubacterium* *Catenabacterium* and *Ramibacterium* were first established by *Prevot* (20) and included into Bergey s Manual in 1957 The division into 3 genera is mainly based upon morphological criteria which makes the exact classification difficult since the morpho logy within these genera is varying The genus *Lactobacillus* is subdi vided into 15 species However as pointed out in Bergey s Manual it is impossible to make an entirely satisfactory differentiation of the spe cies in this genus on account of inadequate comparative data (4)

Therefore since none of these 4 genera seems to be satisfactorily

differentiated we will first compare some of the main properties of this group of microorganisms with those found in our strains

The species included in these 4 genera may broadly be divided into 2 groups—the homofermentative and the heterofermentative—although this distinction is not clear cut As shown our strains act heterofermentatively upon carbohydrates Of the 48 species listed in the 4 genera mentioned 27 are said to break down carbohydrates heterofermentatively and only these species will be discussed

TABLE 9

Comparison of Some Properties Found in Our Strains and in the Heterofermentative Species Listed in the Genera Eubacterium Catenabacterium *and* Ramibacterium

Microorganisms	Property				
	Liquefaction of gelatin	Effect upon milk	Reduction of nitrate to nitrite	Production of H S	Production of indol
Our strains	—	—	—	+	—
Eubacterium					
E foedans	—	—	+	+	0 §
E niosii	—	+	—	0	—
E obsti	+	—	—	—	—
E r ctale	—	+	—	+	—
E quartum	+	+	—	+	0
E pseudo tortuosum	+	—	+	+	0
E t rtuosum	—	+	—	—	0
E quintum	+	+	0	0	0
E l mosum	+	—	—	—	0
E ethylicum	—	+	—	0	—
E ureolyti um	—	+	+	+	0
E bifo me	—	+	—	—	—
E nit ilogenes	—	—	+	—	—
E aerofaciens	—	+	—	—	—
Catenabacterium					
C helmintho des	+	+	—	+	0
C filamentosum	—	+	—	0	—
C contortum	—	—	—	0	+
Ram bacterium					
R ramosum	—	+	—	0	—
R pleuriticum	—	—	—	+	—
R ramosoi les	—	+	—	0	+
R pseudo ramosum	—	+	—	+	+
R dentium	—	—	—	0	+
R alactolyticum	—	—	—	+	+

Based upon *Bergey s M nual* (4) and *Prevot* (23)
§ The term 0 means no data given

The heterofermentative species in the genus *Lactobacillus* i e *Lactobacillus pastorianus L buchneri L brevis* and *L fermenti* differ distinctly from our strains in both physiological and biochemical proper

ties (susceptibility towards air effect upon milk fermentation of trisaccharides and higher carbohydrates etc.) (4 24)

In the group of 23 species left in the tribe *Lactobacilleae*, the results reported for the liquefaction of gelatin effect on milk reduction of nitrate to nitrite and production of hydrogen sulphide and indol were compared with the results found in our strains As may be seen from Table 9 14 out of these 23 species differ from our strains in respect of 2 or more of these 5 tests The remaining 9 species will be further discussed

Eubacterium foedans produces fetid odour forms nitrite from nitrate and produces acetylmethylcarbinol (4 23) Fetid odour is also found in *E mosu*, which in addition coagulates milk and ferments lactose (4 23) *F ethylicum* coagulates milk and ferments glycerol and lactose (4 23) *Catenabacterium filamentosum* coagulates milk ferments lactose and forms acetylmethylcarbinol (4 23) *C contortum* occurs in long twisted chains of 30 or more elements produces indol and acetylmethylcarbinol (4 23)

Ramibacterium pleuriticum shows the same results for the 5 tests mentioned in Table 9 as our strains However the strains in this species show a pleomorphic microscopic appearance with many bifid forms (3 4 23) their production of hydrogen sulphide is weak (4 23) or may be absent (3) They are not thermoresistant (23) and are obligate serophilic on isolation (23) Further they are not able to ferment arabinose and xylose (3) and most often also unable to ferment maltose and saccharose (4 23) *R dentium* and *R alactolyticum* both show bifid forms they form indol and acethylmethylcarbinol (4 23)

Thus all the species listed in these 4 genera *Lactobacillus* *Catenabacterium* *Eubacterium* and *Ramibacterium* differ in some way from our strains either morphologically physiologically or biochemically However the results obtained for our strains strongly suggest that they belong to the tribe *Lactobacilleae* In addition to the properties already mentioned other findings in our strains such as non motility (4 23) absence of catalase and oxidase production (6) tendency towards anaerobic requirements (4 6 23) vitamin dependency (6 19 27) susceptibility to antibiotics (13) and non pathogenicity to mice (4 23) etc are often found among the species listed in this tribe Therefore in view of all the similarities it is likely to conclude that our strains may be regarded as members of the tribe *Lactobacilleae*

Further evidence for this opinion has been produced recently (16) In a separate study strains obtained from other laboratories and belonging to 21 species in the tribe *Lactobacilleae* were investigated for their capacity to transform bile acids Of the 24 strains investigated 14 were able to split conjugated bile acids and 7 strains all belonging to the genus *Eubacterium*, were capable of oxidizing the hydroxyl group at C 3 or C 7 The results concerning the heterofermentative species in the genera *Eubacterium* *Catenabacterium* and *Ramibacterium* are

shown in Table 10 However none of the 24 strains investigated was capable of 7α dehydroxylating chenodeoxycholic acid or cholic acid This reaction which is a major feature in the intestinal bile acid transformation makes it important to attempt to identify our strains When more strains belonging to species in the tribe *Lactobacilleae* have been investigated the capability to transform bile acids especially 7α dehydroxylation may be taxonomic characters of great value and thus clarifying the problems involved in assigning our strains a definite place in the system of classification

TABLE 10

Bile Acid Metabolism in Heterofermentative Strains in Eubacterium Catenabacterium *and* Ramibacterium (16)

Microorganisms	Splitting of bile acid conjugates	Formation of derivatives of chenodeoxycholic acid with TLC mobilities of		
		(3α–OH 7 keto)	(3–keto 7α–OH)	(3α–OH)
E moniliforme	+	—	—	—
E tortuosum	—	—	—	—
E quintum	+	+	—	—
E aerofaciens	—	—	—	—
C helminthoides	+	—	—	—
R ramosum	+	—	—	—
R pleuriticum	—	—	—	—
R dentium	+	—	—	—
R alactolyticum	+	—	—	—
Our strains	—	+	+	+

TLC = Thin Layer Chromatography

SUMMARY

Eight strains of anaerobic Gram positive non sporeforming rods isolated from rat and human fecal sources and capable of 7α dehydroxylating bile acids were investigated On the basis of their morphological physiological and biochemical properties it was concluded that they could be regarded as members of the tribe *Lactobacilleae* A definite identification within a specific species was however not possible

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heparin 5000 IU/ml without preservative (Heparin Vitrum) s c in two equal doses + 0 15 ml of Streptopenin (KABI) containing 0 25 g streptomycin and 200 000 IU benzyl penicillin/ml i m

II 12 male rabbits which were given daily 7 5 mg Cortal i m + 0 15 ml Streptopenin i m

III 5 rabbits 3 males and 2 females which were given daily 1 ml Heparin 5000 IU/ml without preservative s c in two equal doses + 0 15 ml Streptopenin i m

IV 3 male rabbits which were given daily 0 15 ml Streptopenin i m

All injections were at 8 a m except the second heparin injection which was at 4 p m

During the experiment the animals were given daily 130 g Ewos pellets or the same quantity of a hay mixture The composition of the two types of food was found to be very similar The animals were given free access to water

Biochemical methods The day before the experiment began and also after 7 21 and 35 days and on the last day of the experiment 5 ml of venous blood were taken in heparinized tubes after 12 hours starvation After immediate cooling in iced water the samples were centrifuged the plasma frozen and analyzed within 3 weeks for its content of total cholesterol phospholipids and triglycerides 1 ml plasma was extracted with chloroform and methanol as described previously (*Carlson* 1963) The total cholesterol was determined by Tschugaeff s colorimetric method (*Hanel* & *Dam* 1955) The phospholipids were determined as organic phosphorus and the triglycerides as triglyceride glycerol (*Carlson* 1963) During the 46th day of the experiment the variation of LPA was determined under non fasting conditions 5 ml venous blood were taken in test tubes containing 0 5 ml 0 1 M sodium citrate immediately before the injections at 8 a m and then 1 4 8 9 13 and 24 hours later The samples were placed immediately in iced water and centrifuged The plasma was pipetted off and deep frozen and within 3 weeks LPA determination was performed as follows The plasma was incubated *in vitro* with a substrate solution according to a method described previously (*Boberg* & *Carlson* 1964) The activity was expressed as the quantity of liberated free fatty acids per time unit and ml plasma during the incubation The fasting blood sugar was tested every 7th day according to the glucose oxidase method (KABI) An intravenous glucose tolerance test was performed on a number of animals during the final week 0 5 g glucose/kg body weight was injected as a 30 per cent solution into the marginal vein of one ear after 24 hours fasting After 5 10 20 30 60 and 180 minutes blood samples were taken from the marginal vein of the other ear and these were analysed by the glucose oxidase method The K value for the elimination was determined (*Ikkos* & *Luft* 1957) The presence of glucose and ketone bodies in the urine was determined by means of the Ames series for rapid determination Clinistix and Ketostix During the last week of the experiment the plasma creatinine concentration was determined (*Loken* 1954) The body weight was checked weekly

Morphological methods Immediately after the animals had been killed a complete autopsy was performed Tissue pieces were fixed immediately in 5 per cent neutral formalin Ca formalin and Bouin s fixation medium Staining method used were (*Romeis* 1948) Mc Manus PAS phosphotungustic acid hematoxylin (PTAH) hematoxylin eosin Laidlaw s stain for elastin Wilder s stain for reticulin van Gieson s stain Congo Red and methylviolett For the demonstration of fat Sudan Black B and Oil Red O were used and also Baker s stain for phospholipids For the quantitative analyses of the experimental lesions described below a PAS stained 5 μ thick cross section from the central part of a kidney from each animal was used Glomeruli with experimental lesions as described below were counted and expressed in per cent of the total number of glomeruli counted in the section The statistical calculations were performed according to current methods (*Snedecor* 1962)

RESULTS

During the experiment 6 animals died 5 from the Cortal heparin group and 1 from the Cortal group Thus in the following the results

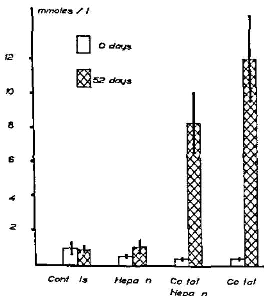


Fig 1

The plasma triglyceride concentration before the commencement of treatment (0 days) and at the end of the treatment period (52 days) in the four groups controls (n 3) heparin treated (n 5) Cortal + heparin treated (n 11) and Cortal treated (n 11) Mean ± s e m

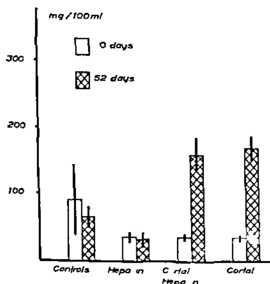


Fig 2

The plasma cholesterol concentration before the commencement of treatment (0 days) and at the end of the treatment period (52 days) in the four groups controls (n 3) heparin treated (n 5) Cortal + heparin treated (n 11) and Cortal treated (n 11) Mean ± s e m.

from 11 animals from both of these groups 5 animals from the heparin group and 3 animals from the control group will be reported

Biochemical results The plasma lipid values in the heparintreated animals and the controls did not change significantly during the experimental period The rabbits which had been treated with Cortal + heparin developed a massive lipaemia during the experimental period with an increased plasma concentration of all lipid fractions such as choles

heparin 5000 IU/ml without preservative (Heparin Vitrum) s.c. in two equal doses + 0.15 ml of Streptopenin (KABI) containing 0.25 g streptomycin and 200 000 IU benzyl penicillin/ml i.m.

II 12 male rabbits which were given daily 7.5 mg Cortal i.m. + 0.15 ml Streptopenin i.m.

III 5 rabbits, 3 males and 2 females, which were given daily 1 ml Heparin 5000 IU/ml without preservative s.c. in two equal doses + 0.15 ml Streptopenin i.m.

IV 3 male rabbits which were given daily 0.15 ml Streptopenin i.m.

All injections were at 8 a.m. except the second heparin injection which was at 4 p.m.

During the experiment the animals were given daily 130 g Ewos pellets or the same quantity of a hay mixture. The composition of the two types of food was found to be very similar. The animals were given free access to water.

Biochemical methods. The day before the experiment began and also after 7, 21 and 35 days and on the last day of the experiment 5 ml of venous blood were taken in heparinized tubes after 12 hours starvation. After immediate cooling in iced water the samples were centrifuged, the plasma frozen and analyzed within 3 weeks for its content of total cholesterol, phospholipids and triglycerides. 1 ml plasma was extracted with chloroform and methanol as described previously (*Carlson* 1963). The total cholesterol was determined by Tschugaeff's colorimetric method (*Hanel & Dam* 1955). The phospholipids were determined as organic phosphorus and the triglycerides as triglyceride glycerol (*Carlson* 1963). During the 46th day of the experiment the variation of LLA was determined under non fasting conditions. 5 ml venous blood were taken in test tubes containing 0.5 ml 0.1 M sodium citrate immediately before the injections at 8 a.m. and then 1, 4, 8, 9, 13 and 24 hours later. The samples were placed immediately in iced water and centrifuged. The plasma was pipetted off and deep frozen and within 3 weeks LLA determination was performed as follows. The plasma was incubated in vitro with a substrate solution according to a method described previously (*Boberg & Carlson* 1964). The activity was expressed as the quantity of liberated free fatty acids per time unit and ml plasma during the incubation. The fasting blood sugar was tested every 7th day according to the glucose oxidase method (KABI). An intravenous glucose tolerance test was performed on a number of animals during the final week. 0.5 g glucose/kg body weight was injected as a 30 per cent solution into the marginal vein of one ear after 24 hours fasting. After 5, 10, 20, 30, 60 and 180 minutes blood samples were taken from the marginal vein of the other ear and these were analysed by the glucose oxidase method. The K value for the elimination was determined (*Ikkos & Luft* 1957). The presence of glucose and ketone bodies in the urine was determined by means of the Ames series for rapid determination: Clinistix and Ketostix. During the last week of the experiment the plasma creatinine concentration was determined (*Loken* 1954). The body weight was checked weekly.

Morphological methods. Immediately after the animals had been killed a complete autopsy was performed. Tissue pieces were fixed immediately in 5 per cent neutral formalin, Ca formalin and Bouin's fixation medium. Staining methods used were (*Romeis* 1948): Mc Manus PAS, phosphotungustic acid hematoxylin (PTAH), hematoxylin eosin, Laidlaw's stain for elastin, Wilder's stain for reticulin, van Gieson's stain, Congo Red and methylviolett. For the demonstration of fat Sudan Black B and Oil Red O were used and also Baker's stain for phospholipids. For the quantitative analyses of the experimental lesions described below a PAS stained 5 µ thick cross section from the central part of a kidney from each animal was used. Glomeruli with experimental lesions as described below were counted and expressed in per cent of the total number of glomeruli counted in the section. The statistical calculations were performed according to current methods (*Snedecor* 1962).

RESULTS

During the experiment 6 animals died, 5 from the Cortal heparin group and 1 from the Cortal group. Thus in the following the results

from 0 40 ± 0 06 to 8 42 ± 1 75 mM per litre In the animals which had been treated with Cortal alone the plasma cholesterol rose from 42 ± 5 to 169 ± 19 mg per 100 ml the phospholipids from 73 ± 5 to 282 ± 29 mg per 100 ml and the triglycerides from 0 36 ± 0 05 to 11 90 ± 2 43 mM per litre

The LLA which was determined in all rabbits during the 46th day can be seen in Fig 4 In the Cortal group and the controls this activity was very small In the Cortal heparin group and the heparin group however the activity was increased after the two heparin injections this response being greater in the former group

The fasting blood sugar values in the two Cortal treated groups showed a very moderate increase In 3 out of 11 animals in the Cortal heparin group and in 6 out of 11 animals in the Cortal group blood sugar values of over 120 mg per 100 ml were noted while no blood sugar value exceeded 200 mg per 100 ml The glucose tolerance curve showed a K value smaller than 0 95 in 2 out of 5 animals in the Cortal heparin group and in 5 out of 7 animals in the Cortal group while in the 3 controls this value exceeded 1 05

Glucose was noted in the urine in the two Cortal treated groups after approximately 5-7 days but disappeared or decreased after a further few days After this time a small varying occurrence of glycosuria was noted No large difference was observed between the two Cortal treated groups with regard to glycosuria The animals treated with heparin alone and the controls had normal blood sugar values and never showed glucose in the urine No positive evidence of ketone bodies was found in any of the animals

TABLE 1

Changes in Body Weight and Weights of Kidney Liver and Adrenal in the Four experimental Groups Mean ± s e m

	Cortal+Heparin	Cortal	Heparin	Controls
Change in body weight in %	−19 7 ± 2 1	−15 0 ± 1 6	+12 8 ± 1 3	+5 2 ± 1 7
Kidney weight g/kg body weight	9 9 ± 0 6	7 8 ± 0 9	7 1 ± 0 5	5 4 ± 0 5
Liver weight g/kg body weight	70 8 ± 3 6	67 1 ± [illegible]5	37 1 ± 3 5	[illegible]3 8 ± 0 7
Adrenal weight mg/kg body weight	100 5 ± 13 1	89 1 ± [illegible] 6	112 2 ± 8 9	13[illegible] 3 ± 0 9

Morphological results A significant reduction in body weight was noted in the two groups treated with Cortal while in the animals of the remaining groups there was an increase (Table 1)

The weight of the kidneys in the three first groups was significantly greater than of those in the control group Further the kidneys of the

animals treated with Cortal + heparin were greater in weight than those of the animals which were given Cortal alone (Table 1)

The most marked change observed in the microscopic examination of the kidneys in the Cortal heparin and Cortal groups was the presence of hyaline masses in the glomeruli (Fig 5) These were usually round—oval and had a diameter of up to half the glomerular diameter The smaller masses were usually crescent shaped and lay on the opposite side of the vascular pool on the convex part of a capillary loop These masses of hyaline material will be called in the following experimental lesions according to *Bloodworth & Hamwi* (1955) The exact position of the hyaline material in relation to the different components of the capillary wall could not be established with great certainty by light microscopy particularly where the larger experimental lesions were concerned In many cases the mass appeared to be situated in the lumen of the vessel while in other cases it seemed to be entirely localized to the space of Bowman s capsule In places the capillary loops were seen to be adherent to the parietal surface of Bowman s capsule especially where experimental lesions were present The lesions exhibited a homogenous or somewhat clustered appearance and red blood cells were often seen enclosed in the hyaline material A glomerulus would sometimes contain two or even three such lesions (Fig 6) but the great majority of glomeruli however contained none As regards staining the experimental lesions were eosinophilic and strongly PAS positive On reticulin staining (Wilder) small black scales were seen but no structures resembling reticulin fibres With PTAH staining large areas were seen in the experimental lesions which assumed a blue tone like that of fibrin The lesions which covered capillary loops as crescent shaped masses assumed a stronger positive fibrin stain (Fig 7) In isolated experimental lesions a moderate occurrence of lipids was seen with fat staining (Fig 8) while the staining of phospholipids with Baker s stain was negative The amyloid staining similarly gave negative results In

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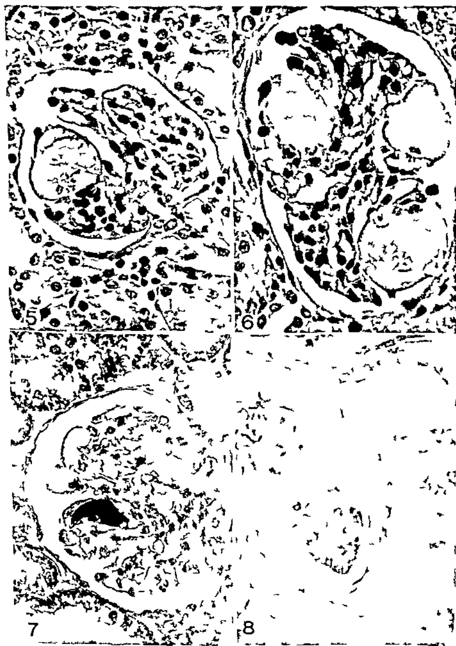
Figs 5–8

Fig 5 PAS stained section from animal treated with Cortal + heparin In the peripheral part of a capillary loop an experimental lesion is seen encl sed by the capillary basal membrane outside which epithelial cells can be observed On the concave side the capillary lumen is seen to contain blood cells which are also observed enclosed in the hyaline material

Fig 6 Glomerulus containing three experimental lesions of somewhat varying positivity to PAS Cortal + heparin

Fig 7 PTAH stained section from animal treated with Cortal + heparin A fibrin positive lesion is seen covering a peripheral capillary loop and resembles the so called fibrin caps that are observed in human diabetes The peripheral capillary loops are dilated

Fig 8 Fat stained section (Sudan Black B) from animal in the Cortal + heparin group showing sudanophilia in an experimental lesion


5
6
7
8

isolated glomeruli capillary loops containing strongly sudanophil material resembling fat emboli were seen but these were not associated with any experimental lesions

In the quantitative analysis of the experimental lesions (Table 2) it was found that the animals which had been treated with Cortal + heparin had a higher frequence of glomeruli containing such lesions than those treated with Cortal alone Qualitatively no difference in the experimental lesions was seen between the Cortal heparin and the Cortal groups In the other two groups no such lesions were seen The difference in the frequency of experimental lesions between the Cortal heparin group and the Cortal group was statistically significant (P<0 001)

TABLE 2

The Percentages of Glomeruli Containing Experimental Lesions Mean ± s e m in the Four Experimental Groups

	Cortal+Heparin	Cortal	Heparin	Controls
Frequency of experimental lesions	6 47 ± 1 59	0 5 ± 0 16	0 00	0 00

The glomeruli exhibited capillary dilation which in parts was very pronounced Usually the cell nuclei were seen to be pycnotic and shrunken and the cytoplasm swollen The basal membrane appeared to be intact

The blood vessels in the kidneys showed no changes apart from some prominence and increased PAS positivity in the macula densa in the two groups treated with Cortal Cylinders of both the hyaline and the granular type were seen in the tubuli of the animals of these two groups In the other two groups no cylinders were seen In the two Cortal treated groups several tubuli were seen to be dilated a number of such dilations being observed immediately proximal to the cylinders

A peculiar change was observed in the tubular epithelium in several animals in the two Cortal treated groups and the heparin group Occasional tubuli differed from their surrounding by showing vacuolization of the epithelium so that in extreme cases the nucleus and cytoplasmic components were displaced laterally and the cell assumed an appearance resembling a signet ring (Fig 9) The vacuoles contained neither fat nor glycogen and showed no dubbelrefraction in polarised light

The livers of the animals in the two Cortal treated groups were greatly enlarged (Table 1) and exhibited both macroscopic and microscopic fat deposits The livers of the heparin treated animals were also significantly enlarged but showed no fat deposits on fat staining In the lungs of the animals in the Cortal treated groups fat staining showed abundant lipid masses in the capillaries and also in medium sized vessels The adrenal glands in the two Cortal treated groups weighed

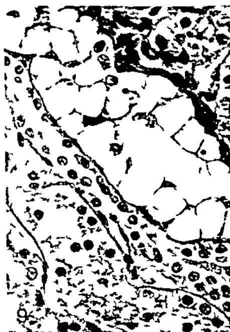


Fig 9
In an isolated tubule the cells are greatly vacuolized and have assumed the appearance of a signet ring PAS stained section from animal treated with Cortal + heparin

less than those in the control group (Table 1) No arterial or venous thromboses were discovered anywhere in any of the animals and neither was any bleeding seen apart from at the injection sites

DISCUSSION

The morphology in cortisone nephropathy in the rabbit has been described in detail (*Rich et al* 1950 *Rosen et al* 1954 *Bloodworth & Hamwi* 1955 *Wilens & Stumpf* 1955 *Moran et al* 1962 *Wilson et al* 1962 *Ogilvie et al* 1965) and our results are in essential agreement with previous findings

To call the hyaline masses in the glomeruli occurring after the administration of cortisone in the rabbit nodular lesions which is usual in the literature seems rather inadequate when considering that this term is used for the classical Kimmelstiel Wilson noduli in human diabetic nephropathy As many investigators have pointed out the glomerular changes induced by cortisone in the rabbit constitute no experimental counterpart to these noduli We prefer instead to use the term experimental lesions according to *Bloodworth & Hamwi* (1955)

It has been established by means of electron microscopy that the hyaline material in the glomeruli in cortisone nephropathy in the rabbit is situated mainly within the capillaries and also that the capillary wall shows degenerative changes (*Moran et al* 1962 *Ogilvie et al* 1965) It seems highly probable from the intracapillary location the

positive fibrin staining and the presence of blood cells in the material together with the demonstration of albumin globulin and fibrin in it with fluorescent antibody methods (*Moran et al* 1962) that the changes represent capillary thromboses *Moran et al* claim to be able to support the hypothesis that cortisone nephropathy in the rabbit corresponds directly to the exudative lesions in human diabetic nephropathy They base this hypothesis on their electron microscopic findings and on the large occurrence of globulin in the material in both conditions The exudative lesions in human diabetic nephropathy were first described by *Barrie et al* (1952) and differ primarily from Kimmelstiel Wilson noduli by their homogenous structure and positive fibrin staining On the other hand *Ogilvie et al* consider that these conditions are not identical since the exudative lesions in human diabetes are mainly situated between the endothelium and the basal membrane while the material in cortisone nephropathy in the rabbit lies within the capillary

It is worthy of observation that in this experiment we obtained a potentiation of the cortisone nephropathy in the rabbit by simultaneous administration of heparin while *Rosen et al* report an inhibition The conditions in our experiment and theirs differ only with regard to the length of experiment which was 21 days in their case compared to 52 days in ours The cortisone dose was the same and the heparin dose which we used was the same as that for which they report a complete inhibition of the renal changes This descrepancy in the results might be explained by the fact that the coagulation inhibiting effect of heparin is at first sufficient to prevent the formation of intracapillary thrombosis In the long run however another potentiating effect of heparin becomes predominant

It is known that corticosteroids *in vitro* give rise to an increased mobilization of free fatty acids (FFA) from adipose tissue in the rat (*Fain et al* 1963) This effect is believed to run parallel with the glucocorticoid action of the different steroids As far as is known no corresponding investigations have been made on the rabbit If we assume a similar mechanism the great decrease of adipose tissue the pronounced lipaemia and the deposition of fat in different organs *e g* in the liver in our cortisone treated rabbits could be explained *Rich et al* (1951) report a raised plasma concentration of FFA in cortisone treated rabbits *Day & Peters* (1958) who measured the LLA in plasma by means of change in optical density state that cortisone has an inhibitory effect on the LLA in plasma after heparin injection In our experiments the LLA was significantly higher in the animals treated with cortisone + heparin than in those which received heparin alone

The aetiology of cortisone nephropathy in the rabbit has been subject to discussion *Wilens & Stumpf* (1955) consider that some disturbance in the lipid metabolism would be of importance pathogenetically and support this assumption on the fact that after the administration of cortisone the rabbit is the animal that developed the most severe

lipaemia and is also the only animal in which renal changes of this type had been induced by cortisone *Berdjis* (1960) however has since seen similar changes after cortisone treatment in the rat In addition *Wilens & Stumpf* have observed fat in the renal lesions and in adjacent capillaries Our results do not support the possibility that the renal lesions could be caused by a cortisone induced lipaemia expressed as a high triglyceride concentration as such since the animals in our experiment which received both cortisone and heparin and which developed the most severe renal damage had a lower plasma concentration of triglycerides

We suggest the possibility that the cortisone nephropathy in the rabbit may be caused by an increased mobilization of FFA The explanation for the potentiation of the cortisone nephropathy with heparin would then be that the heparin by activating LLA further increases the turnover of FFA in the plasma by hydrolysis of the large quantities of circulating triglycerides FFA have been found to give rise to an increased tendency to coagulation The administration of long saturated fatty acids both *in vivo* (*Connor et al* 1963) and *in vitro* (*Connor* 1962) has given rise to thrombosis Even after endogenous mobilization of FFA by ACTH in the rabbit widespread thrombosis has been observed (*Hoak et al* 1962) Another possibility is that the raised concentration of FFA in our experiment gave rise to the kidney lesions via direct endothelial damage and that the capillary thrombosis occurred as a secondary phenomenon

The mobilization of FFA is also increased in human diabetes Therefore in the light of the morphological similarities which exist between the cortisone nephropathy in the rabbit and the exudative lesions in human diabetes a common etiology in these two conditions seems conceivable This might explain the fact that the exudative lesions in human diabetes occur mainly with inadequately controlled diabetes where coma episodes occur when the lack of insulin and thus the FFA mobilization is more pronounced

SUMMARY

Cortisone treatment for 52 d[illegible] in rabbits gave rise to hyaline homogeneous apparently intracapillary situated masses in the renal glomeruli By the simultaneous administration of heparin a higher frequency of such changes was observed In animals treated with heparin alone no such changes occurred

During the experiment the concentration of phospholipids total cholesterol and in particular triglycerides in the plasma rose to high levels which were somewhat higher in animals treated with cortisone alone than in those which received cortisone + heparin No effect on these lipid fractions was noted in animals treated with heparin alone

The plasma lipoprotein lipase activity rose considerably after the

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The plasma lipoprotein lipase activity rose considerably

injections in the heparin treated and cortisone + heparin treated animals the increase being greatest in the latter No increase was noted in the animals treated with cortisone alone

The possible effects of these changes in the lipid metabolism on the pathogenesis in cortisone nephropathy in the rabbit are discussed

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SIMULTANEOUS GENERALIZED CYTOMEGALIC INCLUSION DISEASE AND PNEUMOCYSTIS CARINII PNEUMONIA

A Case of a 2 Year 5 Month Old Girl

By

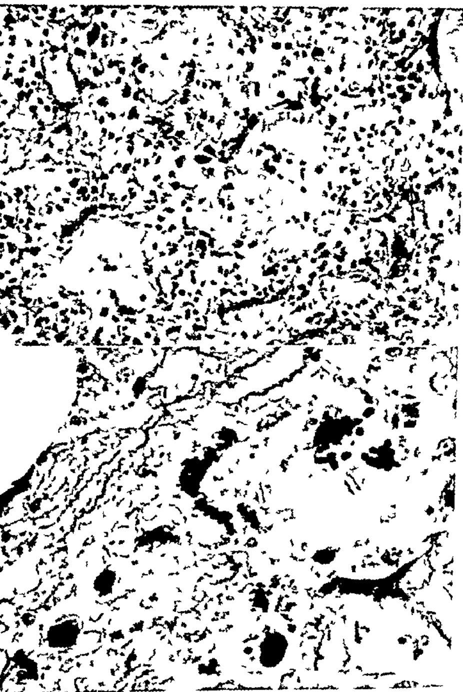
KARI T MAATTA

Received 20 xii 66

Pneumocystis carinii pneumonia and generalized cytomegalic inclusion disease occur simultaneously more often during the first 6 months of life (*e g Ahvenainen* 1952 and *Hamperl* 1956) than later in infancy Among adults the condition is rare (*e g Hamperl* 1956 *Symmers* 1960 and *Kramer et al* 1962) In Scandinavia a few cases of generalized cytomegalic inclusion disease have been described (*Ahvenainen* 1952 *Bjorklund & Wiebert* 1959 *Robertson & Sundelin* 1962 and *Thorell & Nathorst Windahl* 1965) as well as pneumocystis carinii pneumonia in older infants or after infancy (*Nathorst Windahl et al* 1964 and *Hakulinen & Maatta* 1964) Only once before a simultaneous occurrence of these two diseases in an infant has been presented in Scandinavia (*Ahvenainen* 1952) The present paper describes a rare case of simultaneous generalized cytomegalic inclusion disease and pneumocystis carinii pneumonia in a 2 year 5 month old girl

CASE HISTORY

This female infant weighed 870 grams at birth two weeks before the estimated time of delivery She was hospitalized eight days old because of a luxation of the right and subluxation of the left hip The baby was hospitalized again at the age of two months for respiratory infection and diarrhea *Salmonella typhi murium* was cultured from the stools Because of this and recurrent respiratory infections the patient stayed at the hospital continuously over a year At the age of one and a half years also a bilateral otitis was diagnosed in addition to the respiratory infection and chronic diarrhea (the stool cultures were negative for salmonellas the stool analysis being normal) Thereafter the child was nursed at an orphanage for about half a year During this time she occasionally had fever and diarrhea which however cured by antibiotic therapy From the orphanage the child was admitted to a hospital when her general condition grew worse Attacks of diarrhea had occurred more frequently during the two months before the hospitalization In addition to the diarrhea a bilateral otitis was diagnosed at the hospital *Pseudomonas aeruginosa* was cultured from the ears In X ray pictures of the thorax the hilar markings were enlarged and those of the perihilar structures fibrously accentuated Both the mental and physical development of the patient were retarded (the age of development 11–12 months) Chromosome examinations paperchromatography of



g 1 Lung In the middle foamy material and hyaline membrane in alveolar spaces scant mono tic infiltration in interstitium in the lower left alveolar epithelial cells ith inclu ions (Haemat xylin (iesin × 160)

g 2 Lung In the upper right man bla k pneumoc tis carinii organisms In the lower left two al eolar epithelial cells with irus in lu ions black too (Co mori methenamine sil er × 400)

the urine amino acids and cerebrospinal fluid findings were normal Blood cultures were negative and serum protein concentration was 6 1 grams/100 ml Immuno electrophoresis was not done neither cytologic or virologic examinations to exclude cytomegalia Clinically the patients disease remained etiologically obscure Sepsis or an inborn error of metabolism besides respiratory infection and diarrhea were considered most probable The child was transferred into an oxygen tent but died in increasing cyanosis and dyspnea

Autopsy Findings

The autopsy was performed two days after death The body was 71 cm long and weighed 7300 gr The pleural cavities were free from adhesions and fluid The lungs were fleshy solid throughout and all specimens from different lobes sank in water At the distal end of the ileum six small ulcerations were found The left tympanic cavity was full of pus The rest of the organs were grossly normal Bacteriologic or virologic samples were not taken

Microscopically the lungs were strongly hyperemic but there were no actual hemorrhages Hyaline membranes lining the walls of alveoli alveolar ducts and respiratory bronchioles were seen abundantly The alveolar epithelial cells were swollen and cuboid Some alveoli were filled with eosinophilic foamy material In several alveolar spaces there were large cells (obviously alveolar epithelial cells) with inclusions in the nucleus and cytoplasm The interstitium was scantly infiltrated by lymphocytic and large monocytic cells Only very few plasma cells were seen separately in the interstitium (Fig 1) With Gomori s methenamine silver stain several black pneumocystis carinii organisms were demonstrated in the alveolar lumina Partly also the virus inclusions in the alveolar epithelial cells appeared darkly stained (Fig 2) In the specimens of the small intestine the epithelium was lacking and the submucosa was profusely infiltrated by polymorphonuclear leukocytes Inclusions could be seen both in the nucleus and cytoplasm of many vascular endothelial cells (Fig 3) The kidney specimens showed a few tubular epithelial cells with inclusions (Fig 4) Very rarely calcium appeared in the tubules In pancreatic tissue inclusion containing cells were found in one islet of Langerhans (Fig 5) No cytomegalic cells were seen in the acini Several ganglionic cells of the brain showed inclusions both in the nucleus and cytoplasm In addition a few solitary cytomegalic cells were found in the liver spleen thyroid gland adrenal glands and mesenteric lymph nodes but not in the heart No acid fast bacilli were seen in lung tissue

DISCUSSION

The present case can be compared with those in which either pneumocystis carinii pneumonia or generalized cytomegalic inclusion disease has been diagnosed as the terminal infection of a malignancy This child had a prolonged *salmonella typhi murium* infection which lasted

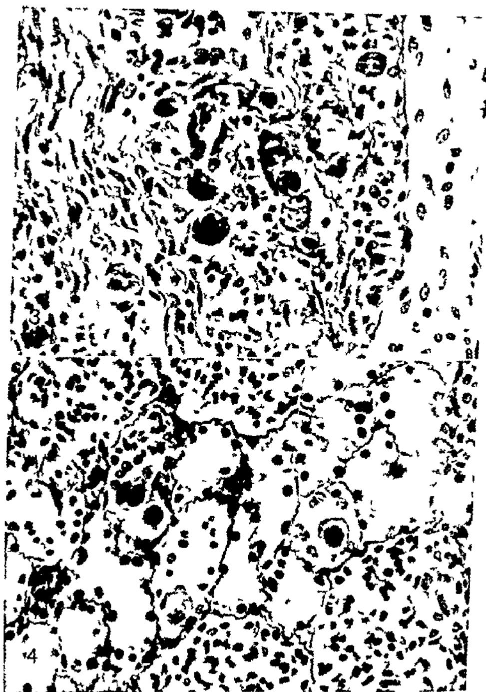
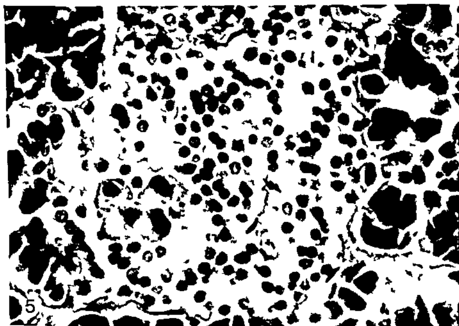


Fig 3 Ileum Endothelial cells of blood vessel with inclusions in the cytoplasm and nucleus (Haematoxylin v Gieson × 400)

Fig 4 Kidney Tubular cells with nuclear and cytoplasmic inclusions (Haematoxylin v Gieson × 400)



Fig

Pancreas An islet of Langerhans In the central left a few cytomegalic cells with cytoplasmic and intranuclear inclusions bodies with halo (Haematoxylin v Gieson × 400)

over a year and in addition respiratory infections which diseases can be considered to have markedly lowered the patients general resistance Furthermore the relative lack of plasma cells in the pulmonary interstitium suggests hypogammaglobulinemia such as *Kramer et al* have presented combined with pneumocystis carinii pneumonia and generalized cytomegalic inclusion disease The fact that two weakly virulent diseases which usually occur in infants were diagnosed to occur simultaneously after infancy presumes remarkably lowered resistance in this case caused by severe and prolonged infections

SUMMARY

A rare case of simultaneous generalized cytomegalic inclusion disease and pneumocystis carinii pneumonia in a 2 year month old girl is presented The child had been almost all her life under hospital care for recurrent respiratory infections and diarrhea caused by *salmonella typhi murium* lasting over a year The most severe affections of generalized cytomegalic inclusion disease appeared typically of the age of the child in the lungs and intestine In addition a few solitary cytomegalic cells were found in the liver pancreas (only in an islet of Langerhans) spleen adrenal glands brain thyroid gland kidney and

vascular pattern of induced primary hepatic carcinoma in rats by means of microangiographic and histologic methods

MATERIALS AND METHODS

Induction of Liver Tumours

35 Sprague Dawley rats were given 0 04 per cent of the hepatocarcinogenic substance 3 methyl 4 dimethylaminoazobenzene (3 Me DAB) in their fodder a semi synthetic diet consisting of 12 per cent caseine 79 per cent cane sugar 5 per cent corn oil and 4 per cent salts and vitamins This diet was deficient in riboflavin in order to potentiate the carcinogenic effect The average daily intake was 13 g so *the amount of carcinogen per rat per 24 hours was some 5 mg*

After 8 months on this diet 7 rats were still free from cancer although they did exhibit more or less pronounced cirrhosis of the liver and a few small cysts The remaining 28 rats had altogether 184 liver tumours varying in diameter from 1 to 50 mm All these tumours were examined microangiographically and histologically

Methods

The rats were laparotomized under ether anaesthesia The widest possible catheter was introduced into the portal vein of 17 and into the aorta of 18 rats The portal vein in the latter rats was ligated in the hilus and the aorta proximal to the origin of the coeliac artery 0 5 ml of heparin (Vitrum 5000 I U /ml) was infused via the catheter followed by 1 ml of a 2 per cent Xylocaine ® solution (Astra) which caused the rats to die Then a finely granulated suspension of contrast medium—consisting of equal volumes of 30 per cent barium sulphate suspension (Micropaque Powder Howards) and 10 per cent formalin solution—was injected into the portal vein at a pressure of about 25 mm Hg and into the aorta at a pressure of about 125 mm Hg The hepatic veins were opened and blood mixed contrast medium exuded for some seconds after the commencement of the contrast injection

After the injection of contrast medium the liver was removed the vessels to the lobes were ligated and the lobes were separated After fixation in formalin and paraffin embedment the preparations were cut into slices 50 to 1000 μ thick which were X rayed (Kodak maximum resolution plates and/or Typon Typolith TL-PE film Machlett AFG 50 X ray tube 15–20 mA 15 20 kV 1–15 mins focus film distance 42 cm) Contact microangiograms only were made Stereomicrograms were obtained by angling the specimen and film holder 4 to 8 between consecutive exposures The pictures were examined in a Zeiss mirror stereoscope

For microscopy 8 μ sections were cut from the X rayed paraffin slices They were stained with haematoxylin eosin and haematoxylin v Gieson

Tumour Histology

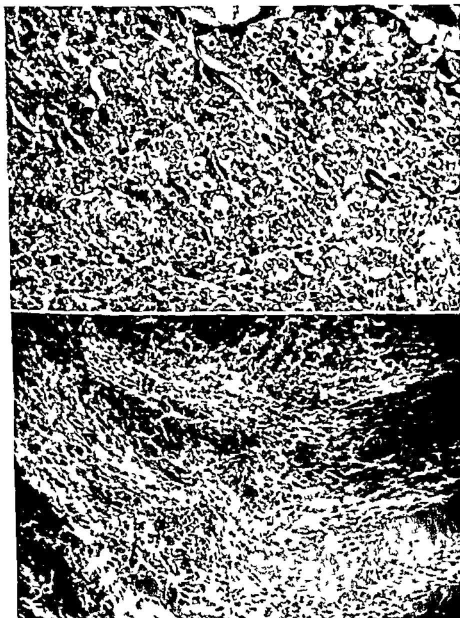
The 184 induced primary malignant tumours comprised 66 hepatocellular carcinomas 62 cholangiocellular carcinomas 46 mixed hepatocellular and cholangiocellular carcinomas and 10 undifferentiated tumours The hepatocellular and cholangiocellular carcinomas were classified into well differentiated and poorly differentiated carcinomas

Arteriograms were obtained of 43 hepatocellular carcinomas 37 cholangiocellular carcinomas 30 mixed and 6 undifferentiated tumours Portograms were obtained of 23 hepatocellular 25 cholangiocellular 16 mixed and 4 undifferentiated tumours

RESULTS

Arteriography

Hepatocellular carcinoma The afferent vessels to the tumours were branches of local arteries They were wider than normal and dislocated by the tumour They rather abruptly branched out in the tumour tissue The vessels in well differentiated tumours (Fig 1) formed a fairly



Figs 1 2

Fig 1 Well differentiated hepatocellular ca inoma × 1?8 Haematoxylin v Gie son

Fig 2 Arteriogram of the same tumour as in Fig 1 Finely m shed n twork of si nusoid like vessels × 34



Figs 5–6

Fig 5 Arteriogram of the same tumour as in Fig 4 Irregularly branching uniformly wide slender vessels × 34

Fig 6 Portogram Small well differentiated hepatocellular carcinoma with sinusoid like vessels × 34

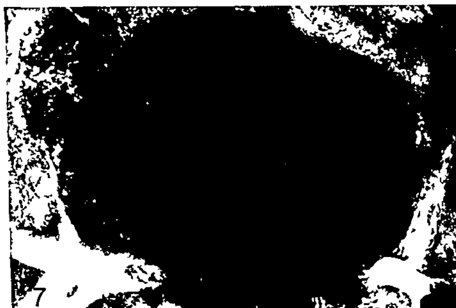


Fig 7

Portogram Cholangiocellular carcinoma lacking contrastfilled vessels × 34

filled deformed and dislocated portal vessels were encountered as well in the central parts of the tumour In exceptional cases a little contrast medium had entered vessels originating from such persisting portal branches

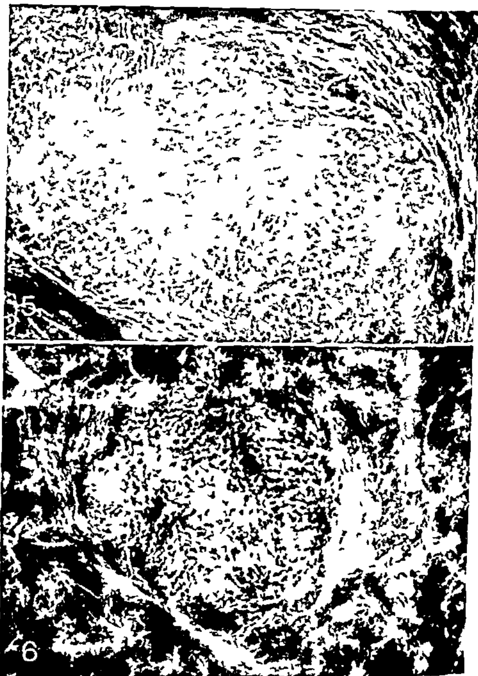
Cholangiocellular Carcinoma As a rule no contrast medium entered the tumour vessels not even those in the marginal parts (Fig 7) More or less dislocated local portal branches and sinusoids were nevertheless observed in the tumours Contrast medium from such portal branches only rarely entered the tumour vessels

Mixed hepatocellular and cholangiocellular carcinoma Here and there the tumours exhibited sporadic contrast filling of wide sinusoid like profusely anastomosing vessels and of occasional persisting more or less deformed coarser portal vessels Elsewhere there was no contrast filling of newly formed vessels but persisting portal vessels and sinusoids were filled Comparative histological examination revealed that the former regions were of hepatocellular and the latter of cholangiocellular type

Undifferentiated tumours The vessels in undifferentiated tumours showed no filling with contrast medium

DISCUSSION

Injection experiments of the type described here are extremely difficult to conduct under wholly physiological conditions Hence the results



Figs 5–6

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EFFECT OF HEPATIC ARTERY LIGATION ON INDUCED PRIMARY LIVER CARCINOMA IN RATS

Preliminary Report

By

L A V NILSSON and L ZETTERGREN

Received 2 ii 67

The fact that cholangiocellular carcinoma completely and hepatocellular carcinoma for the most part are supplied by the hepatic artery prompts the question whether regressive changes can be induced in such tumours by ligation of this vessel (For references see *Nilsson & Zettergren* 1967) *Markowitz* stated in 1952 It is possible that secondary carcinoma of the liver may be treated by ligation of the hepatic artery However no attempts to treat malignant liver tumours by ligation of the hepatic artery have apparently been made except experimentally on a few rabbits with Vx carcinoma (*Breedis & Young* 1954) No regression was found

The present paper will report preliminary results of an investigation with the aim of studying the effect of ligation of the hepatic artery on induced liver carcinoma in rats

MATERIAL AND METHODS

Adult Sprague Dawley rats were used Liver carcinoma was induced by feeding a standard semisynthetic diet containing 0 04 per cent 3 methyl 4 dimethylaminoazo benzene After 4 to 5 months the rats were laparotomized under ether anaesthesia The hepatic artery in 19 animals with liver tumours was ligated in the hilus The number of visible tumours their size and position were recorded Of the 19 rats one was killed on the 5th day after the operation 7 on the 7th 5 on the 14th 4 on the 21st and 2 on the 28th day The liver was taken out and fixed in formalin solution The tumours and surrounding liver tissue were embedded in paraffin sectioned and stained with haematoxylin eosin and haematoxylin van Gieson About equally large liver tumours chosen at random from nonligated rats were used as controls with a view to estimating the incidence of spontaneous regressive changes

Altogether 28 superficially situated liver tumours were observed at the operation their diameters varying between 2 and 3 mm At autopsy 17 additional tumours were found Most of these were very small and situated deeply in the parenchyma Thus 45 tumours were available for microscopic examination

This investigation was supported by grant 62 4 1 from the Swedish r Society



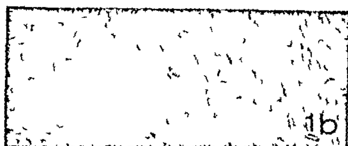


Fig 1a Necrobiotic cholangiocellular carcinoma 5 days after ligation of hepatic artery The tumour is unstained owing to the necrobiosis × 5 Haematoxylin—v Gieson

Fig 1b Enlargement of the tumour in Fig 1 The pattern appears as a shadow × 180 Haematoxylin—v Gieson

RESULTS

Gross Observations

All rats survived ligation of the hepatic artery and seemed to be unaffected by it except one rat which seemed to be ill and was killed on the 5th day The 3 tumours in this rat were of necrotic appearance but of the same size as at ligation

In rats killed on the 7th and 14th day many tumours also appeared to be necrotic and some of them had decreased in size Most of them had altered colour the previously greyish tumours had now changed into yellowish or greenish The tumourfree liver tissue exhibited no gross changes

In rats killed on the 21st and 28th day after the ligation some of the previously observed tumours were also necrotic while others appeared to be vital A few necrotic tumours had decreased in size In some rats fibrous adhesions had developed between the liver surface or superficially situated hepatic tumours and neighbouring organs and tissues such as stomach duodenum diaphragm abdominal wall and omentum



Fig 2
Necrotic tumour 7 days after ligation of the hepatic artery × 5 Haematoxylin—v Gieson

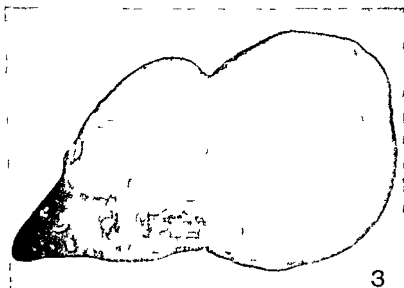
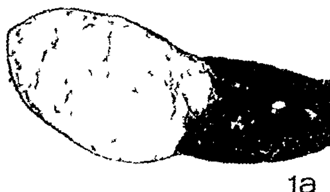


Fig 3
Necrotic hepati tumour 14 1 y after ligati n of the h patic a t ry As in Fig 2 the tumour is unstained owing t the necr si × 5 H ematoxyl n— G on



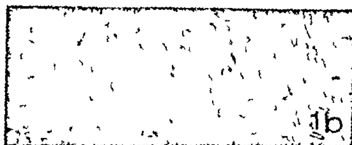


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All rats survived ligation of the hepatic artery and seemed to be unaffected by it except one rat which seemed to be ill and was killed on the 5th day The 3 tumours in this rat were of necrotic appearance but of the same size as at ligation

In rats killed on the 7th and 14th day many tumours also appeared to be necrotic and some of them had decreased in size Most of them had altered colour the previously greyish tumours had now changed into yellowish or greenish The tumourfree liver tissue exhibited no gross changes

In rats killed on the 21st and 28th day after the ligation some of the previously observed tumours were also necrotic while others appeared to be vital A few necrotic tumours had decreased in size In some rats fibrous adhesions had developed between the liver surface or superficially situated hepatic tumours and neighbouring organs and tissues such as stomach duodenum diaphragm abdominal wall and omentum



Fig 2
Necrotic tumour 7 days after ligation of the hepatic artery × 5 Haematoxylin—Gieson

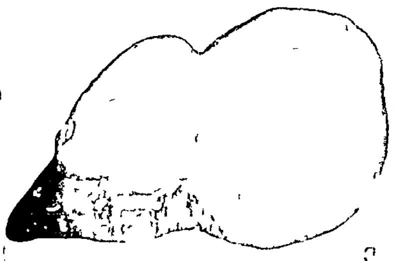


Fig 3
Necrotic hepatic tumour 1[illegible] [illegible] after ligation [illegible] the hepatic artery As in Fig 2 [illegible] the tumour is unsta[illegible] [illegible]ing to the n[illegible]cr[illegible] Haematox[illegible]lin

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LEIOMYOBLASTOMA OF THE STOMACH

A Clinico Pathological Study of 10 Cases

By

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Received 17 iv 67

Martin et al (1960) reported 6 cases of tumours which they called myoid tumours of the stomach *Stout* (1962) reported 69 tumours of the same microscopic structure and proposed the name *leiomyoblastoma* The microscopic structure of this type of tumour is dominated by rounded or polygonal cells without smooth muscle fibres The cytoplasm may be acidophilic *Stout* (1962) suggests that the gradual transition sometimes visible between the rounded cell areas and leiomyoblasts of more normal appearance points towards an origin in smooth muscle tissue This is however doubted by *Martin et al* (1965) The nuclei are often completely or partly surrounded by a clear zone which does not stain with PAS nor with fat stains The mitotic rate is low If the mitotic rate is high a malignancy may be indicated (*Stout* 1963 *Fontaine et al* 1964 *Kay* 1964 *Gupta & Chandler* 1965 *Martin et al* 1965 *Sautot et al* 1965 *Guillet & Feroldi* 1965 *Schoefield & Fox* 1965 *Herrington* 1966) The reports are summarized in Table 1 which shows that there are not many data on follow ups Most of the reported tumours were considered to be benign Martin et al reported one malignant out of 6 Stout reported 2 malignant out of 69

The sparseness of follow up data in other reports seemed to us to warrant the publication of the following report of 10 cases these include the first one diagnosed in Finland [1]

MATERIAL AND METHODS

The material was collected from the files of the Finnish Cancer Registry and the pathology laboratories in Finland it covers the period 1953 to 1966 inclusive The slides were stained with haematoxylin vanGieson periodic acid Schiff (PAS) haematoxylin eosin Mallory s phosphotungstic acid haematoxylin Masson s trichrome stain and Gomori s silver impregnation for reticulin All available clinical data were collected with the aid of the civil registry authorities and it was checked whether the patients were alive or dead at the time of the survey If patients had died the cause of death entered on the death certificate was noted

This study was supported by grants from *Sigrid Jusélius Foundation*

[1] Case Number 8 operated on by *B L Lindstrom* and diagnosed by *H Teir*

TABLE 1
Summary of Reports on Leiomyoblastomas of the Stomach

	Year of publication	Number of cases	Behaviour benign	Behaviour malignant	Follow up period (years)
Martin et al	1960	6	5	1	1–13
Stout	1962	69	67	2	1–11
Yarington & Cook	1962	1	1		Died on the 4th postoperative day
Sommariva & De Feo	1963	1	1		No data
Fontaine et al	1964	1	1		No data
Kay	1964	1	1		5 5/12 and 4 1/12
Guillet & Feroldi	1965	1	1		3/12
Gupta & Chandler	196	1	1		1
Martin et al	1965	3	3		No data
Sautot et al	1965	1	1		3
Shoefield & Fox	1965	2	2		2 and 11
Herrington	1966	1	1		No data
Tallquist et al	1967	10	9	1	11 years to 3 months

The histological slides were re examined and tumours not corresponding to the histological criteria given above by *Martin et al* (1960) and *Stout* (1962) were excluded from the material All tumours had been removed b surgery

RESULTS

The clinical and pathological data are summarized in Table 2

Age and sex The age of the patients varied between 26 and 71 years the arithmetical mean being 51 9 years There were 6 women and 4 men

Symptoms and signs In 6 patients the tumour was found at laparotomy performed on account of abdominal diseases unrelated to the tumour three cases of cholelithiasis (gall stones) two of peptic ulcer and one case of hiatus hernia In these patients the symptoms and signs could not be evaluated in relation to the tumour In the remaining 4 patients the symptoms were considered to be related to the tumour the period during which symptom [illegible] manifested varied from 2 to 16 weeks before operation was performed

These 4 patients complain d of weakness dyspnea and vertigo they had considerable melaena and haemoglobin values between 3 3 and 8 3 g/100 ml None had haematemesis Two patients had vomited in one of them the tumour situated in the pyloric region interfered with the emptying of the stomach Two patients had slight upper abdominal pain which could be related to the tumour One patient had suffered a slight loss in weight

In three patients the tumour was palpable as a movable mass in the upper abdomen

TAI

Summary of the Clinical and Pathological Findi

Number of cases	Age (years)	Sex	Symptoms and signs	X ray diagnosis	Site of tumour	Treatment
1	71	♂	Weakness Melaena Vomiting Hgb 5.1 g	Gastric carcinoma	Corpus posterior wall	Billroth I resection
2	53	♀	None (chance finding)	Deformation of bulbus duodeno The tumour not detected	Corpus anterior wall	Billroth II resection
3	26	♀	Melaena Hgb 3.3 g Lose of weight. Palpable tumour	Gastric tumour Retention in the stomach	Praepyloric region	Billroth I resection Postoperative X ray therapy
4	59	♂	None (chance finding)	Gastric ulcer The tumour not detected	Corpus lesser curvature	Excision Postoperative X ray therapy
5	40	♀	None (chance finding)	Not examined	Corpus anterior wall	Billroth II resection
6	61	♂	Slight abdominal pain Melaena Hgb 8.3 g Palpable tumour	Gastric carcinoma	Praepyloric region	Billroth II resection Postoperative X ray therapy
7	59	♀	Vomiting Palpable tumour Obstruction of the pylorus	Not examined	Pylorus	Billroth II resection
8	36	♀	Melaena	*Gastric ulcer* The tumour not detected	*Corpus* posterior wall	Excision Actinomycin D
9	52	♂	None (chance finding)	Hiatus hernia The tumour not detected	Corpus anterior wall	Excision
10	6[illegible]	♀	None (chance finding)	Not examined	Corpus anterior wall	Excision

n 10 Cases of Leiomyoblastoma of the Stomach

Cro s patl ology	Microscopic pathology						Follow up findings length of follow up	Primary histopathological diagnosis
	Infiltrating	Rounded cells dominating	Clear cells dominating	Clear cells occasional	Pleomorphism	Number of mitoses in 50 h p f		
wo tumours 6×6 m and 0 5×0 7 cm oft expansive ntramural and ntragastric crater cm in d ameter	?	+	+	—	—	2	Died 3 weeks after operation	Myosarcoma
×1 cm intramura irm expansive	+	+	—	+	—	1	Living 11 years 6 months	Myoma malignum
×4 cm intramural nd intra astric rater 1 5 cm deep	+	+	—	+	—	3	Living 10 years 9 months	Schwannoma malignum
5×1 3 cm ntran ural well imited, expansive	+	+	—	+	—	0	Living 10 years 3 months	Neurinoma malignum
×2 cm subserous vell limited	?	+	+	—	+	3	Living 6 years 11 months	Leiomyoblastoma malignum
5 cm firm xpansive 8 cm crater	?	+	—	—	+	0	Living 6 years	Leiomyosarcoma
×6 cm firm odulous mucosa tact	+	+	—	+	++	3	Living 4 years 6 months	Leiomyosarcom
Three tumours) intramural 2×3 m 0 8 cm crater)3) pedunculous ubserous cm in liameter	+	+	—	+	+−	?	[illegible] m ta tases [illegible] s 3 onth	Neuro arcoma et neurinoma Later le omyoblastoma
×2 5 cm susErous ell limited.	?	+	+	−	+	0	L ing 1 ear 3 month	Leiomyoblastoma
5×0 6 cm ub erous firm xpan ive	+	+	+	—	+	0	Li ng 3 m nths.	Leiom oma atypicum.

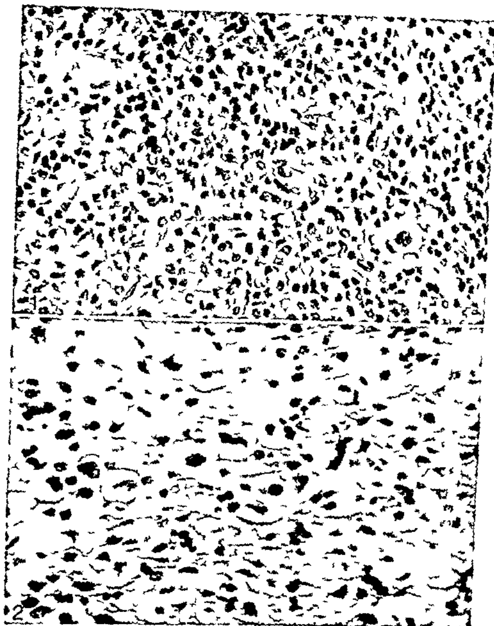


Fig 1 Leiomyoblastoma dominated by rounded or polygonal cells without visible smooth muscle fibers Haematoxylin van Gieson 400 ×

Fig 2 *Leiomyoblastoma dominated by clear zones surrounding the nuclei Haema*toxylin van Gieson 400 ×

Radiological findings Stomach radiography was *performed in 7 pa*tients and the tumour measuring more than 4 cm in diameter in all these cases was diagnosed by this means in three patients *In two of* these the X ray diagnosis was malignant tumour of the stomach

in one patient tumour of the stomach was diagnosed without comment to malignancy.

Therapy. In 6 patients the tumour was removed by resection of the stomach: Billrooth I in two cases and Billrooth II in 4 cases. Two patients were given additional radiotherapy. In 4 patients the tumour was removed by excision. One patient received Actinomycin D infusions two years later when liver and lymph node metastases were found at a second laparotomy (case report number 8).

Gross pathology. In 7 patients the tumour was situated in the corpus region: in 4 in the anterior wall of the stomach, in two in the posterior wall and in one in the lesser curvature. In two patients the tumour was found in the prepyloric region and in one patient in the pylorus. All ten tumours were well defined as intramural expansively growing, rounded, ovoid or lobulated masses. No capsule could be seen. The diameter of the tumours varied between 0.5 and 6 cm. The mucosa often showed ulceration and crater formation. In two patients the tumour was multiple: one showed two tumours, one three tumours.

The consistency of the tumour was mostly described as firm, myoma-like, but in one case as soft.

Microscopic pathology. In four cases the microscopic specimen consisted exclusively of tumour tissue and did not contain any parts of the stomach wall. In 6 cases the tumour was partly encapsulated and partly showed microscopic infiltration into the surrounding smooth muscle tissue. All tumours were rather cellular and dominated by rounded or polygonal cells without visible smooth muscle fibers (Fig. 1). In five cases the nuclei surrounded by typical clear zones were dominant (Fig. 2), in five cases these appeared occasionally. Two cases were characterized by a pronounced nucleic pleomorphism (Fig. 3). In four cases the pleomorphism was considered modest and in four cases the nuclei were rather uniform.

The number of the mitotic figures were counted in 50 random high power fields. In four cases no mitoses were found, in one case there was one, in two cases two and in three cases three mitotic figures in 50 high power fields.

The pathology of case number 8 deserves special consideration. Two years after excision of the tumour of the stomach laparotomy was performed because of endometriosis and metastases to the liver and ligamentum hepatoduodenale were found. The microscopic structure (Fig. 4) of the metastases was characterized by high cellularity and pronounced pleomorphism and did not differ from that of the primary tumour. Two mitotic figures were found in 50 random high power fields in both the primary tumours and in the metastasis.

Initial histo-pathological diagnosis. In 8 cases the primary histopathological diagnosis was that of a malignant myogenic or neurogenic tumour. The tumours diagnosed as benign were found in 1962 or later.

Follow up. One patient died 3 weeks after operation (case number



Fig 3 Leiomyoblastoma with pleomorphic nuclei occasionally surrounded by clear zones Haematoxylin van Gieson 400 ×

Fig 4 Liver metastasis of leiomyoblastoma Note the high cellularity and the pronounced pleomorphism Haematoxylin van Gieson 400 ×

1) Nine patients were alive at the time of survey including the one patient whose tumour had metastasized The patient is living and is well 1½ years after metastases to the liver were found No metastases or recurrences were found in any other patients

The clinical data pathological findings and follow ups are summarized in Table 2

CASE REPORT

Case 8 (138155)

The patient was a woman of 36 who came for examination for dyspnea on exertion and weakness Severe haemorrhagic anaemia was diagnosed X ray examination of the stomach revealed a high dorsal ulcer of th corpus The ulcer did not disappear by conservative therapy and surgery was performed in June 1962 Three separate tumours about 2 5 cm apart were located in the posterior wall of the stomach The one at the most cranial site 2 × 3 cm in diameter was intramural the other two each about 2 cm in diameter were pedunculated subserous The intramural tumour grew intragastrically and had an ulcer measuring 0 5 × 0 6 cm in the mucosa The tumours were excised in a single block Histological diagnosis established on the basis of a frozen section was neurinoma and of a paraffin section neurosarcoma and neurinoma (*H Teir*) 1 month later the diagnosis of leiomyoblastoma was made Gynaecological laparotomy was performed on the patient in May 1964 on account of endometriosis and a metastasis was palpated on the side of the lesser curvature of the stomach Re exploration showed that the stomach was intact but both lobes of the liver were sites of metastases of about 1 cm in diameter 6 in number They were partly superficial partly embedded Their histological appearance concurred with the picture of the primary tumour All the specimens were also examined by Dr A P *Stout* The patient had received Actinomycin-D infusions in January 1965 The patient is still alive and in good condition (January 1967)

DISCUSSION

This report underlines the fact that this type of tumour forms a distinct entity and deserves a separate name Whether the term myoid tumour of the stomach (*Martin et al* 1960) is used or leiomyoblastoma (*Stout* 1962) seems immaterial The distinctions of these tumours is also underlined by the fact that 8 out of 10 tumours discussed in this report were primarily diagnosed as malignant growths In only one case out of 10 however did the tumour metastasize as far as is known This ratio corresponds more closely to that reported by *Martin et al* (1960) who found one metastasizing tumour among 6 rather than to the ratio reported by *Stout* (1962) who found 2 malignant cases in a series of 69 cases Among these however 6 tumours were found at autopsy and in 11 cases there was no follow up which may have same influence on the ratio The metastases seen in case number 8 were casual findings observed at laparotomy for endometriosis and the patient is subjectively well 1½ years later This implies that even though patients from a clinical point of view seem to be well metastases may be manifest Therefore the evaluation of the behaviour of this tumour needs a very long follow up The clinical findings and the gross pathology is the same as in other myogenic tumours of the stomach This agrees with earlier reports (*Martin et al* 1960 and 1965 *Stout* 1962 *Herrington* 1966)

It should be pointed out that the clinical behaviour of the 10 leiomyoblastomas reported here corresponds closely to the behaviour of those in the group of cellular leiomyoma described by the present writers in

an earlier report (*Salmela & Tallqvist* 1967) where one out of 24 of these cellular leiomyomas had a malignant course although the histopathological findings had suggested its nature

SUMMARY

10 cases of leiomyoblastoma of the stomach are reported follow ups covered periods of from 3 months up to 11 years A histopathological diagnosis of malignancy had been established in 8 cases out of 10 One out of 10 tumours metastasized the patient concerned is well and alive two years after the metastases were found

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ULTRASTRUCTURE OF THE RENAL TUBULES IN ACUTE RENAL INSUFFICIENCY

By

T STEEN OLSEN

Received 28 ii 67

In acute anuric renal insufficiency (lower nephron nephrosis tubular necrosis shock kidney acute ischaemic anuria etc) there is a total functional break down but only scant structural lesions are seen by light microscopy of the renal tissue So far patho physiological studies in patients have not succeeded in explaining the functional basis of the disease

The light microscopic picture can only be evaluated with confidence from a material of biopsies because even a slight autolysis makes the interpretation of details uncertain The lesions described by *Brun* (1954) and *Brun & Munck* (1957) on the basis of a biopsy material were dilated distal convoluted tubules with flattened epithelium and often containing pigmented casts interstitial oedema and small foci of cellular infiltration Hydropic changes of the epithelium of the proximal tubules were considered to be due to infusions of hypertonic fluids or Dextran Contrary to the common opinion tubular necrosis was not characteristic for the lesion

Only a few electron microscopic studies of human material from patients suffering from acute renal insufficiency have been published (4 5 6 9 14) These were all done on a relatively few patients using a technique (methacrylate) now considered inferior

Comparison with normal human material is of great importance not least in the study of the renal tubules where artificial changes caused by the technical procedur[illegible] [illegible] ble The aim of this report is to present a controll[illegible] study of the fine structure of the renal tubules during oliguri[illegible] Another pape[illegible] will describe the ultrastructure of the glomeruli 13)

Aided by the *Danish Scien[illegible]ndation*
Presented at the III Intern[illegible]nal Congress [illegible] Nephrology Washington sept 1966
I am indebted to Mr *A L[illegible]h Andersen* Graduat[illegible] Engineer for [illegible]luable advice and to laboratory technic[illegible]n Mr *Kaj Peters[illegible]n* for the photo[illegible]raphic reproductions. Chief surgeon *H S[illegible]joldb[illegible]rg* made the kidney [illegible]op[illegible]es

TABLE 1

Case	Biopsy No	Sex	Age	Clinical data	Duration of oliguria (< 400 ml/ day)	Approximate duration of polyuria (> [illegible]000 ml/ day)	Dialysis treatment	Death (d) or recovery (r)
H S	9	♂	61	*Choledocholithotomy—pancreatitis*—septicemia—shock	10	—	+	d
F M	4	♂	[illegible]9	Oliguria following sulphonamide therapy	7?	8	+	d
I M	1	♂	43	Acute barbiturate poisoning—shock	7	—	+	d
B BJ	2 10	♀	47	*Gun shot wound in the abdomen*—lesions of the bowel—shock	19	—	+	d
A N	5	♂	4	Truxal poisoning (Truxal = chlorprothixeni a tranquilizer)	5	11	—	r
C J	8	♀	29	Post partum *haemorrhage—shock*	3	7	—	r
O M	11	♂	[illegible]	Oliguria following urethral dilatation Pt in long term treatment with Delcortin Acute adrenal insufficiency?—shock	11	2	+	r
R N	[illegible] 12	♂	[illegible]5	Traumatic amputation of the leg—shock	12	[illegible]0	+	r
J J	7	♀	5[illegible]	Chole locholithiasis—jaundice	[illegible]	4	+	r
I L	3	♂	55	Perforated duodenal ulcer—peritonitis —shock	3	—	+	d

MATERIAL AND METHODS

12 biopsies from 10 patients were taken at different times after the development of oliguria the earliest from the second day the latest from the 22nd day Two of the biopsies were made in the polyuric phase and one in the transition between oliguric and polyuric phases The most important clinical data appear from Table 1 Laboratory data and infusions related to each biopsy are summarized in Table ?

Control material Biopsies from 4 subjects were procured in the same way as those from the patients In these cases urinalyses and creatinine clearances were normal and the individuals had never had complaints from the urinary system They were hospitalized on account of appendicitis ruptured intracranial aneurism haemolytic anaemia (no haemoglobinuria the biopsy was performed during splenectomy) and early stage cancer of the cervix Light microscopy showed normal structure The ages of these patients ranged between 12 and 33 years

The biopsies from the patients with oliguria were performed under anaesthesia The right kidney was exposed by a small incision in the lumbar region A tissue cylinder 10 to 30 mm long was obtained with a Vim Silverman needle by suction The biopsies from the control patients were obtained from the anterior surface during laparotomy for other purpose One of the controls was biopsied by cutting out a small wedge of renal tissue from the left kidney at the moment of death Artificial circulation and respiration was performed until the moment of biopsy The right kidney from this patient was used for transplantation

Light microscopy A part of the tissue cylinder was fixed in Helly s fluid mixed just before use from the stock solution and a formaldehyde solution Following fixation which took exactly one hour the tissue was rinsed in distilled water for 12–18 hours followed by dehydration in alcohol treatment with xylol embedding in paraffin Serial sections of a thickness of about 2–3 microns were cut using a rotating microtome Routine stainings were Haematoxylin eosin Sirius red iron haematoxylin periodic acid–Schiff–haematoxylin phosphotungstic acid haematoxylin for fibrin methyl violet congo red and thioflavine (fluorescence) for amyloid

Electron microscopy 10 to 20 small pieces were cautiously cut from each end of the tissue cylinder with a razor blade and immediately fixed by immersion in OsO_4 (biopsy number 4 6 9 and 12) or in glutaraldehyde (the remaining) Three of the controls were fixed in OsO_4 one in glutaraldehyde

Osmic acid fixation was performed for one hour in OsO_4 1 0 per cent in Michaelis buffer (pH 7 3) at room temperature Washing in the buffer *Glutaraldehyde* fixation was in 3 0 per cent glutaraldehyde at room temperature for one hour Rinsing several times in 0 1 molar Cacodylate buffer (pH 7 4) One hour post fixation in 1 per cent OsO dissolved in the same buffer Washing with Cacodylate buffer containing sucrose 0 2 molar Both types of fixation were followed by dehydration in acetone embedding in Vestopal W The specimens were sectioned in a LKB Ultratome stained at the copper grids with uranyl acetate for ½ hour Microscopy with a Philips EM 200

OBSERVATIONS

Light Microscopy (Table 2)

The histological changes in the biopsies corresponded well to those earlier described (1 2) The most apparent lesions were dilatation of the distal tubules with the occurrence of pigmented often haemoglobin like casts and flattening of the epithelium which often presented some degenerative changes Only one case presented unequivocally evidence of patchy necroses of some of the distal epithelial cells The proximal tubules often showed some degree of hydropic change ("osmotic nephrosis) In three cases this lesion was present to a severe degree with vacuolar swelling of the epithelium covering nearly all the proximal tubules represented in the biopsy In these cases some hydropic change could also be observed in other parts of the nephron i e the

TABLE

Biopsy No	Case	Day after onset of oliguria	Serum creatinine biopsy day (mg/100 ml)	Urine volume (ml) biopsy day	Total amount of hyperosmotic glucose iv before biopsy (glucose in grams)	Hyperosmotic glucose iv last day before biopsy (glucose in grams)
1	L.M	2	8.0	100	—	—
2	B.Bj	2	6.9	40	100	100
3	F.L.	3	8.0	10	90	—
4	E.M	4?	10.8	140	90	90
5	A.N	5	16.0	390	200	200
6	R.N	5	10.0	120	100	100
7	J.J	5	8.5	275	410	200
8	G.J	7	11.0	2750	250	250
9	H.S	8	12.0	470	780	200
10	B.Bj	8	6.8	20	930	200
11	O.M	10	10.5	130	740	200
12	R.N	22	3.0	3650	1920	—

Dialysis 1–2 days before
§ Low molecular dextrane (Pharmacia, Sweden)

epithelium covering the capsule of Bowman and the distal tubules. *One of these patients had received 2600 ml 6 per cent Rheomacrodex* 3–5 days before biopsy. By a special technique using periodic acid—Schiff in alcohol instead of water (12) the Rheomacrodex could be demonstrated by a strong PAS positive reaction of the vacuoles. The two other patients had received infusions of hypertonic glucose solution a few hours before biopsy. (This was also the case in other patients but they presented only a slight degree of osmotic nephrosis or none.)—*Necrosis of proximal epithelium was never seen.* The glomeruli were normal in all cases.

Electron Microscopy

Proximal tubules. These can be divided into the convoluted part and the straight part. Each segment reveals some characteristic features

2

Other intravenous infusions before biopsy (ml)	Light microscopy				
	Interstitial cell infiltr	Dilatation of distal tub	Casts	Necrosis of distal epithel	Hydropic change prox tubul
—	+	+	+	0	0
Rheomacrodex§ 1500 ? days before	+	++	++	0	+
Rheomacrodex§ 2600 3 5 days before	+	++	++	0	+++
—	+++	++	+	0	±
—	0	++	+	0	0
Mannitol 300 13 days before— Rheomacrodex§ 500 5 days before	+	++	++	+	+
—	+++	++	++	0	+
—	+	++	++	0	+++
—	+	++	+	0	+++
See above (biopsy No ?)	++	++	+++	0	0
—	0	+	+	0	0
See above (biopsy No 6)	0	+	+	0	0

well known from studies of rats and mice (19) The brush border and the basal infoldings are most numerous and of greater height in the convoluted part The brush border is lower and the microvilli are fewer in the straight part situated in the medullary rays Here also the basal infoldings are lower and fewer and they do not contain regular compartments for mitochondria In both parts vesicles and vacuoles can be identified in the apical cytoplasm

In the *controls* the brush border was occasionally replaced by bulbous protrusions of clear cytoplasm with few or no vesicles and organelles The lumina often contained cellular debris or they were occluded by swelling of epithelial cells The height and amount of the basal infoldings seemed to follow the pattern described in animals though it could be difficult to identify with certainty the part of the proximal tubule which was represented in the section In some tubules

in some electron microscopic sections) The proximal tubules in these biopsies did not show severe lesions (Fig 1) The brush border was well preserved nearly all cytoplasmic details were normal The amount of cytosomes in the cytoplasm was moderately but definitely augmented These cytosomes contained granular or homogenous matter residues of membranes and myelin like formations Cytosegrosomes (7) with identifiable remnants of mitochondria or other cytoplasmic organelles were not observed It was the impression that the basal infoldings generally were reduced but no definite proof of this could be obtained because of the difficulty involved in the problem of determining the exact position of a section of a tubule in biopsy material No necrotic cells were seen Some epithelial cells were rather low with distended lumina *Biopsies with osmotic change* (Nos 2 3 4 6 7 8 and 9) All these biopsies contained a multitude of single membrane bounded vacuolar structures in the cytoplasm of the proximal epithelium evenly distributed apically and basally (Fig 3) No difference in appearance of the vacuoles in patients who had received Rheomacrodex mannitose or glucose could be demonstrated The size was most often between 1 and 3 microns Some of them were optically empty and were similar to the cytoplasmic vacuoles normally seen although of greater size Other contained evenly distributed fine granular material of medium density or clumps of dense material pieces of membranes filaments or myelin like figures thus forming cytosomes Rather often these vacuolar structures were confluent They never anastomosed with the basal labyrinth Sometimes the membranes were ruptured the openings adjoining clear areas of the cytoplasm The intensity of this vacuolar change was very different from tubule to tubule and from cell to cell The basal infoldings were also reduced in these cases Cells with osmotic vacuoles contained normally structured mitochondria perhaps in an amount less than that seen in other proximal epithelial cells No transitional forms between mitochondria and vacuoles or cytosomes were seen The number of dense bodies seemed to be moderately reduced The nuclei were normal and the cells were not necrotic In one biopsy a few dark epithelial cells with densely packed mitochondria ribosomes and vacuoles and pyknotic nuclei were observed These few cells were considered necrotic resembling the anhydropic type of necrosis described in experimental hypoxia (21)

Distal Tubules

The *control subjects* has distal tubules rather similar to those observed in animals Some preparations contained tubul[illegible] which could be identified as the straight part of [illegible] distal tu[illegible] the basal infoldings were high often extendin[illegible] he height [illegible] er level of the nucleus and formin[illegible] compart[illegible] aining [illegible]

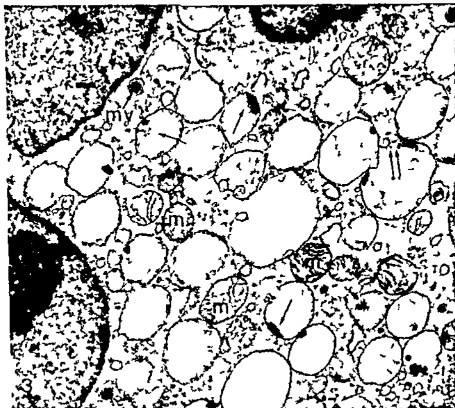


Fig 3

Biopsy No 9 Part of proximal cell with osmotic change Several large vacuoles some of them with dense inclusions (arrows) forming cytosomes Vacuole with rupture of the membrane (double arrow) Mitochondria (m) often showed degeneration of matrix type but this was also observed in controls mv multivesicular body Osmic acid fixation [illegible]0000 ×

larly oriented mitochondria Most sections from distal tubules were thought to represent the convoluted part Here the basal infoldings were lower the mitochondria round and without constant relation to the folded basal cell membrane At this site as well as in the proximal tubules the mitochondria sometimes had an expanded clear matrix

The luminal surface of the cells was flat with only few and short microvilli The cytoplasm contained evenly distributed dense bodies but only a few cytosomes The apical cytoplasm contained vesicles and small vacuoles The basal labyrinth was always small slitlike The nuclei were regular vesicular with finely granulated chromatin and contained nucleoli Sometimes the chromatin was condensed at the periphery Some sections revealed the presence of large confluent electron dense masses of a homogenous structure and with sharp

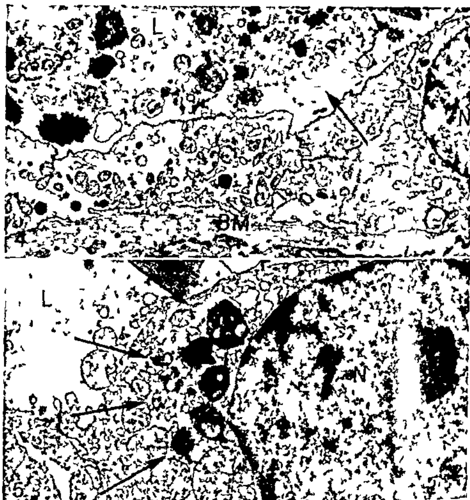


Fig 4 Biopsy No 7 Flattened distal tubular cells with disappearance of microvilli and reduced basal infoldings Cast in the lumen (L) with dark granular material (haemoglobin?) remnants of cellular organelles (mitochondria cytosomes) and filamentous material (fibrin?) (arrow) BM basement membrane N nucleus Glutaraldehyde fixation 9500 ×

Fig 5 Biopsy No 6 Luminal part of distal tubular cell with several cytosomes (arrows) to the left of the nucleus (N) Also here the mitochondria seem to be degenerated but the same changes could be observed in control L lumen Osmic acid fixation 20000 ×

boundaries Inside were big empty vacuoles These structures could represent lipoid droplets (lipofuscin?)

The distal tubules from three patients with oliguria occasionally contained osmotic vacuoles resembling those observed in the proximal part of the nephron They were fewer and smaller (Nos 2 6 and —more questionably—No 8) The nuclei were usually well preserved in all biopsies and only occasionally some were slightly shrunken with folded nuclear membranes The cells were flat The lumina were

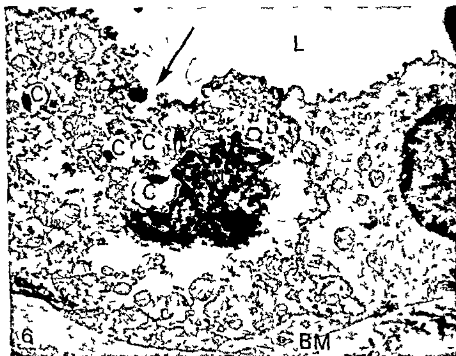


Fig 6

Biopsy No 2 Distal tubule with a necrotic cell (hydropic type of necrosis) adjoining one which has preserved. N pyknotic nucleus in necrotic cell. The cytoplasm is hydropic and contains several cytosomes (C) often with ruptured membranes. Ruptured surface membrane of the cell (arrow). BM basement membrane. L lumen. Glutaraldehyde fixation 13000 ×

distended and contained casts in which the following constituents could be identified 1) irregular, not membrane bounded, electron dense masses, perhaps haemoglobin 2) fragments of epithelial cells 3) free organelles, most often cytosomes and mitochondria and finally 4) finely fibrillar material structured like fibrin. The cytoplasmic constituents did not present severe lesions. Vesicles, mitochondria and endoplasmic reticulum showed normal structure. In three biopsies lipoid droplets were observed. One of the biopsies showed foci of a fibrillar material in most of the distal cells, a phenomenon not seen in the controls or in the remaining patients with anuria. The fibrils were similar to those which normally are present in epithelial cells in the glomeruli. The amount of cytosomes was moderately augmented in all anuric patients. Unquestionable cytosegrosomes were not seen. The amount of free ribosomes and granular reticulum seemed to be increased in biopsies from the later phases of the disease (Nos 9, 10 and 12). The basal infoldings were reduced in height and number, the basal labyrinth had always collapsed. The endoplasmic reticulum

was often distended but this feature could also be seen in the controls

Unquestionable necroses were observed in one biopsy (No 2) They were of the hydropic type (21) The cytoplasm was clear desintegrated involving rupture of the membranes of the cell and its organelles The nuclei were heavily pyknotic Characteristically these few necrotic cells were bordered by preserved not necrotic cells (Fig 6) In the specimen prepared for light microscopy from this case no necrosis was observed

Thin Henle Loops Collecting Tubules

In this material these were not represented sufficiently regularly to allow a description of findings The few Henle loops and collecting tubules which were studied did not reveal significant lesions

DISCUSSION

The fixation of small pieces of tissue by immersion cannot be regarded as optimal for the study of the renal ultrastructure Recent studies have shown the superiority of perfusion for the fixation of this organ (10 18) Obviously such a technique cannot be used in work with human biopsies However in the present study control studies of biopsies from 4 subjects without kidney disease showed reasonably good preservation of the tissue

In the evaluation of our biopsies the possibility of preparative artefacts was constantly kept in mind and deviation from presumed normal structure was compared with the tissue from our own 4 control biopsies as well as with the observations by *Ericsson et al* (8) of normal human tubules (embedded in methacrylate) The possibility of changes caused by the composition of the fixation fluid was also considered (10 11)

The glomeruli from patients with oliguria seem to be normal or only insignificantly altered (14) On the other hand severe lesions of the tubular epithelium observed by light microscopy as well as electron microscopy (4 5 6 9 14) have been described We could not confirm this Compared with normal controls and considering the possibility of artefacts the proximal tubules did not reveal severe lesions if osmotic change was not present The distal tubules were

Figs 7–8

Fig 7 Biopsy No 1 Globular electron dense inclusion with clear vacuoles in distal tubule (lipoid inclusion?) Such inclusions can also be found in controls Glutaraldehyde fixation 20000 ×

Fig 8 Biopsy No 9 Eighth day of anuria Urine volume 470 ml on day of biopsy presumably beginning polyuric phase but the patient died 2 days later from septicaemia. Low distal tubular epithelium with increased numbers of ribosomes (arrows) interpreted as sign of regeneration Reduced basal infoldings L lumen BM basement membrane


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THE EFFECT OF VARIOUS FIXATIVES ON THE PRESERVATION OF ACID GLYCOSAMINOGLYCANS IN TISSUES

By

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Received 10 iv 67

The fixation of acid glycosaminoglycans in tissues has been extensively discussed by *Williams & Jackson* (1956) *Baker* (1958) *McManus & Mowry* (1958 1960) *Pearse* (1960) *Barka & Anderson* (1963) *Conklin* (1963) *Szirmai* (1963) and *Socher* (1965) Regarding the choice of the best fixative opinions differ Thus for instance aqueous formalin has been endorsed by some investigators (*McManus & Mowry* 1958 1960 *Socher* 1965) but criticized by others (*Sylven* 1950 *Szirmai* 1963)

The acid glycosaminoglycans exist as sulphated and nonsulphated compounds Evidence is now available that the sulphated acid glycosaminoglycans (keratan sulphate chondroitin 4 sulphate chondroitin 6 sulphate dermatan sulphate heparan sulphate heparin) occur in the tissues covalently bound to protein (*Shatton & Schubert* 1954 *Malawista & Schubert* 1958 *Muir* 1958 *Gerber et al* 1960 *Matthews* 1962 *Buddecke et al* 1963 *Gregory et al* 1964 *Mathews & Cifonelli* 1964 *Seno et al* 1965 *Toole & Lowther* 1965 *Lindahl* 1966 *Roden & Armand* 1966) Whether or not the unsulphated hyaluronic acid also occurs bound to protein is less certain (see *Brimacombe & Webber* 1964 *Schiller* 1966) Finally concerning chondroitin nothing is known in this respect

In histochemical and autoradiographic studies using various fixatives and solvents for dehydration and staining the possibility should not be overlooked that the polysaccharid protein complexes as well as the glycosaminoglycans possibly not bound to p[illegible]rs may be soluble to some extent in the various fluid Th[illegible]s the [illegible]s[illegible]lt obtained in ordinary histochemical and autoradiographic studies [illegible] unreliable due to loss of glycosaminoglycans during the histolog[illegible] procedure

Attempts have been made pr[illegible]iously to obtain information about the

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The nomenclature suggested b[illegible] *B[illegible]la[illegible]s & J[illegible]anlo* (196[illegible]) has been followed

Abbreviations CP[illegible] cetylpyrid[illegible]m (CPC[illegible] cetylpyridinium chloride

This investigation w[illegible]s made p[illegible]ble [illegible]e[illegible]rch grants from the *Swedish M[illegible]dical Research Council* (Project No K67-12X-540-03) and from th[illegible] *U S Public Health Service* (Grant No AM 69145)

The experiment in which the photographic film was dipped half way down into azure A and subsequently exposed to light revealed that the part of the film which had been immersed in azure A was very weakly blackened while the undipped part was heavily blackened. When the film was not exposed to light a very weak blackening of the dipped part was observed but no reduced silver in the undipped part. It is thus clear that the emulsion in some way was altered by the stain.

Quantitative Radiochemical Findings

The effects of the various fixatives on the preservation of S^{35} containing material in the normal and the rachitic growth plates are seen in Tables 1 and 2, respectively.

TABLE 1

The Effect of Various Fixatives on the Preservation of S^{35} Containing Materials in Normal Epiphyseal Plates

Fixation	Time between administration of S^{35} and death (hours)	Unfixed section	After 10 min fixation M ± Γm	t (unfixed 10 min fix.)	After 4 hours fixation M ± Γm	t (unfixed 4 hours fix.)	Number of sections measured
10% aqueous formaldehyde	24	100	66.5 ± 5.1	6.6	64.1 ± 4.1	8.3	10
10% neutral formaldehyde	24	100	72.0 ± 3.2	8.8	70.2 ± 3.4	8.8	10
1% CPC	24	100	95.3 ± 5.8	0.8	98.1 ± 7.3	0.3	10
0.5% CPC in 4% aqueous formaldehyde	24	100	95.1 ± 7.0	0.7	89.6 ± 5.5	1.9	10
0.5% CPC in 4% neutral formaldehyde	24	100	95.1 ± 4.8	1.0	94.9 ± 4.8	1.1	10
Methanol	24	100	101.7 ± 2.4	0.7	102.9 ± 3.2	0.9	10
Carnoy	24	100	102.6 ± 2.0	1.3	100.5 ± 2.4	0	10
Azure A pH 7.0	24	100	101.5 ± 4.5	0.3	103.9 ± 5.3	0.7	5
Azure A pH 2.0	24	100	98.3 ± 2.5	0.7	98.4 ± 3.2	0.5	6

The results are given in per cent of number of counts per unit time of unfixed sections with the number of counts of unfixed sections = 100 per cent.
M = Mean   Γm = Standard error of the mean
$0.05 > P > 0.01$ $0.01 > P > 0.001$ $P < 0.001$

Using sections from animals labelled 24 hours or three days before death there was a highly significant loss amounting 30–40 per cent of the S^{35} containing material in the epiphyseal plates after fixation with

TABLE 2

The Effect of Various Fixatives on the Preservation of S^{35} Containing Materials in Rachitic Epiphyseal Plates

Fixation	Time between administration of S^{35} and death (hours)	Unfixed section	After 10 min fixation M ± Em	t (unfixed 10 min fix.)	After 4 hours fixation M ± Em	t (unfixed 4 hours fix.)	Number of sections measured
10% aqueous formaldehyde	1	100	44 6 ± 3 6	15 4	27 5 ± 1 3	48 1	5
	24	100	59 1 ± 1 8	22 7	59 1 ± 1 0	40 9	5
	72	*100*	*70 6 ± 3 6*	*8 2*	*71 6 ± 2 4*	*11 8*	5
10% neutral formaldehyde	24	100	67 9 ± 1 7	18 9	62 3 ± 2 0	18 9	10
1% CPC	1	100	38 2 ± 3 1	19 9	32 4 ± 1 5	45 1	3
	24	100	93 7 ± 3 9	1 6	95 3 ± 1 3	3 6	5
0 5% CPC in 4% aqueous formaldehyde	1	100	39 8 ± 0 7	86 0	33 7 ± 2 8	23 7	5
	24	100	95 5 ± 0 8	5 6	98 1 ± 2 1	0 9	4
	72	100	97 4 ± 2 2	1 2	97 2 ± 2 4	1 2	5
Methanol	1	100	91 0 ± 10 8	0 8	75 0 ± 8 2	3 1	5
	24	100	95 4 ± 1 5	3 1	97 3 ± 2 4	1 3	10
	72	100	98 7 ± 3 2	0 4	94 7 ± 3 4	1 0	5
Azure A pH 7 0	24	100	92 7 ± 3 1	2 4	97 0 ± 4 8	0 6	5
Azure A pH 2 0	24	100	94 8 ± [illegible] 1	2 5	971 ± 6 2	0 5	5

The results are given in per cent of number of counts per unit time of unfixed sections with the number of counts of unfixed sections = 100 per cent

M = Mean   Em = Standard error of the mean

$0.05 > P > 0.01$ $0.01 > P > 0.001$ $P < 0.001$

both 10 per cent aqueous formaldehyde and 10 per cent neutral formaldehyde No corresponding loss occurred with the other fixatives In the experiments on the material labelled one hour before killing of the animals it was found that after fixation in 10 per cent formaldehyde 1 per cent CPC and 0 5 per cent CPC in 4 per cent formaldehyde a highly significant decrease in radioactivity occurred amounting to about 60 per cent Using methanol no decrease in radioactivity was noted after 10 minutes fixation while a weakly significant decrease of 25 per cent occurred after 4 hours fixation

Tables 3 and 4 give the results of the experiments in which the risk of loss of sulphated glycosaminoglycans during the stages of treatment

thermore similar results were obtained using the rachitic specimens With regard to the preservation of chondroitin sulphate in the tissues this indicates that these fixatives are very suitable

After fixation in methanol no unequivocal decrease of radioactivity was noted when radiosulphate was given 24 hours or earlier before death Thus no significant extraction of chondroitin sulphate had occurred during fixation with this fluid However as is obvious from the following methanol (like other alcohols and dehydrating media) is not a suitable fixative since it does not make the glycosaminoglycans permanently insoluble

Carnoy s fluid has been recommended by *Hale* (1946) for the study of hyaluronic acid by means of his iron method Furthermore it has been suggested earlier that only a slight loss of glycosaminoglycans occurs when Carnoy s fluid is used as fixative (see *Szirmai* 1963) In the present investigation the radiochemical results show that no losses of chondroitin sulphate occurred from the tissues to this fixative

As shown above there was no appreciable extraction of the sulphated glycosaminoglycans during fixation of epiphyseal cartilage in 0 5 per cent CPC in 4 per cent aqueous formaldehyde (acid and neutral) methanol and Carnoy s fluid However fixation also comprises stabilization of tissue components against the subsequent stages of treatment In the present investigation some light has been shed on this problem

When methanol fixed sections were immersed in water a distinct loss of radioactivity (about 25 per cent) and thus of chondroitin sulphate occurred It is evident therefore that methanol (and other alcohols and dehydrating media) will not chemically alter the chondroitin sulphate protein complexes in such a way as to make them permanently insoluble Consequently after fixation with these fluids resolubilization can occur during the subsequent stages of treatment if such material comes into contact with water or water containing media

When sections fixed in Carnoy s fluid were immersed in water a loss of about 10 per cent of the radioactivity was noted This loss was only weakly significant This result suggests that the risk of loss of chondroitin sulphate during the stages of treatment subsequent to fixation is smaller when Carnoy s fluid is used as fixative than when methanol is employed This may be due to the combined effects of acetic acid and alcohol in Carnoy s fluid whereby the acid glycosaminoglycans are precipitated as a complex with the proteins present

As to material fixed in 0 5 per cent CPC in 4 per cent aqueous formaldehyde (acid and neutral) there is no danger of allowing such material to come into contact with water for a short period of time after fixation since the cetylpyridinium glycosaminoglycan complexes are extremely insoluble in water (*Scott* 1955 1960) Thus autoradiographs of frozen sections previously fixed in this fixative can be performed without any risk of loss of chondroitin sulphate

It has been stated that lower alcohols generally solubilize cetylpyridi

nium glycosaminoglycan complexes (*Scott* 1960 *Kelly et al* 1963) In the present investigation sections fixed in 0 5 per cent CPC in 4 per cent aqueous formaldehyde were immersed in 70 per cent ethanol or absolute alcohol No decrease in radioactivity was noted however These results show that tissues fixed in this fixative can be dehydrated in these fluids without loss of chondroitin sulphate Another dehydrating medium dioxane has been recommended by *Kelly et al* (1963) since this cyclic ether as well as acetone chloroform and hexane has been shown not to solubilize cetylpyridinium glycosaminoglycan complexes (*Scott* 1955 1960 *Kelly et al* 1963)

In general the presence of glycosaminoglycans in a tissue is detected by means of cationic dyes after fixation of the tissue In order to overcome the drawbacks involved in the use of fixatives before staining *Szirmai* (1963) has recommended the immediate staining by cationic dye solutions of fresh frozen sections In this way the glycosaminoglycans are precipitated and stained at the same time *in situ*

When this principle was utilized in the present investigation and fresh frozen sections were directly stained by azure A at pH 7 0 or 2 0 the radiochemical results obtained indicated that no loss of chondroitin sulphate had occurred during immersion in these solutions The radiochemical results also showed that no loss of this polysaccharide had occurred during further subsequent immersion in water

Turning now to the radiochemical investigation of the material labelled one hour before death the results show that after fixation in 10 per cent aqueous formaldehyde there was a highly significant decrease in radioactivity amounting to 60 per cent Using methanol there was no decrease in radioactivity after 10 minutes fixation and a weakly significant decrease after 4 hours fixation this decrease was not as marked however as after fixation in the other fluids These results are thought to be due to the presence of large amounts of labelled inorganic sulphate which are insoluble in alcohols but soluble in aqueous solutions Furthermore the results suggest that labelled chondroitin sulphate was present in the epiphyseal plates as early as one hour after injection of radiosulphate since about 33 per cent of the radioactivity was still present in the specimens immersed in 1 per cent CPC this compound being capable of precipitating polyanions but not low molecular weight sulphated precursors to chondroitin sulphate Previous autoradiographs obtained by the ordinary autoradiographic technique have revealed an almost exclusively intracellular uptake of S^{35} one hour after injection of radiosulphate (*Bélanger* 1952 *Engfeldt & Westerborn* 1960 *Hjertquist* 1961) The results are in agreement with the *in vitro* investigations carried out by *Dziewiatkowski* (1962) *Kaviak* (1963) and *Thorp & Dorfman* (1963) These authors showed by means of chemical methods that chondroitin sulphate was present within the cartilage cells and that S^{35} taken up by these cells after incubation of cartilage in a medium containing radioactive sulphate was present in this glycos

aminoglycan The present results are also in agreement with those of *Bostrom* (1952) who isolated radioactive chondroitin sulphate from costal cartilage two hours after injection of radiosulphate

It was found that irrespective of fixatives used with the exception of azure A) the autoradiograms of the normal specimens previously fixed in the various fixatives were in principal similar The same was true for the rachitic specimens Furthermore the uptake pattern of S^{35} did not differ from that observed in earlier studies of normal and rachitic cartilage where the specimens were embedded in paraffin before autoradiography (*Hjertquist* 1961 *Engfeldt et al* 1962) Thus the present results confirm these earlier investigations performed with ordinary autoradiographic methods

Autoradiography using radiosulphate is a valuable method by which to obtain information about the metabolism of the sulphated glycosaminoglycans at the cellular level In general the most convenient way is to obtain autoradiograms of specimens which have been fixed and subsequently embedded in paraffin The present investigation has shown that if such a technique is to be used 0 5 per cent CPC in 4 per cent formalin seems to be the substance of choice for fixation of chondroitin sulphate in the tissues since this fixative did not cause extraction of the glycosaminoglycan from the cartilage Furthermore no resolubilization occurred during dehydration when 70 per cent ethanol and absolute ethanol were used Obviously it would have been interesting to investigate the effects of the other subsequent stages of the histological embedding procedure Owing to technical reasons however this was not possible

With regard to autoradiography the present investigation has also shown that instead of using specimens embedded in paraffin a useful alternative is to make autoradiograms of frozen sections previously fixed in 0 5 per cent CPC in 4 per cent formalin By doing so the risk of loosing chondroitin sulphate during the subsequent procedure is further diminished

In general autoradiographic examination is performed on unstained sections which later may or may not be stained after the autoradiograms have been developed Often however autoradiography is performed on prestained sections It is obvious from the results reported here that azure A is unsuitable as a stain to be used in cases where subsequent autoradiography of the same section is desired This seems to depend on alterations of the emulsion caused by the stain and not on loss of labelled chondroitin sulphate to the water during the stripping film procedure

It is to be expected that chondroitin sulphate as well as other sulphated acid glycosaminoglycans will behave in the same way in the above discussed respects when present in other tissues Keratan sulphate might possibly be an exception since it is soluble in aqueous solutions containing an excess of CPC Furthermore it seems probable

that the observed effects of the various fixatives also hold with respect to the unsulphated acid glycosaminoglycans hyaluronic acid and chondroitin However since hyaluronic acid in contrast to the sulphated acid glycosaminoglycans possibly occurs not bound to protein in the tissues the observed dissolving ability of formaldehyde may be more pronounced due to the fact that formalin is directed against the tissue proteins and the glycosaminoglycans as such are soluble in formalin

As already pointed out the presence of glycosaminoglycans within a tissue is usually detected by means of cationic dye solutions after fixation of the tissue with various fixatives It is obvious from the present investigation that fixatives containing cetylpyridinium ions are not suitable for fixing frozen sections if subsequent staining is desired since such sections did not stain at all with azure A The reason being that the cetylpyridinium ions block such reactive groups as subsequently should react with the stain However according to *Williams & Jackson* (1956) *Kelly et al* (1963) and *Socher* (1965) material fixed in fixatives containing CPC and subsequently embedded in paraffin stains with cationic dyes

SUMMARY

The ability of various fixatives to preserve acid glycosaminoglycans in the tissues was studied quantitatively using the epiphyseal plate as a test tissue and employing a radiochemical method with administration of radiosulphate *in vivo*

To this end normal and rachitic rats were injected with radiosulphate and killed after 1 24 and 72 hours Frozen sections from the epiphyseal plates with adjacent parts of epiphysis and metaphysis were prepared and subjected to radioactivity determination Furthermore autoradiographic and histological investigations were made

The amount of radioactivity in the sections was determined before and after immersion in various fixatives and in azure A In some cases the radioactivity of fixed sections was also measured before and after immersion in water or ethanol In this way the capacity of various fixatives for preserving chondroitin sulphate within the cartilage could be determined The results suggested that 10 per cent aqueous formaldehyde (acid and neutral) extracted chondroitin sulphate to a considerable degree (30 40 per cent) The best fixatives were found to be 0 5 per cent cetylpyridinium chloride in 4 per cent aqueous formaldehyde 0 5 per cent cetylpyridinium chloride in 4 per cent neutral aqueous formaldehyde and 1 per cent cetylpyridinium chloride in water since these fluids did not significantly extract chondroitin sulphate Similar results were obtained for methanol Carnoy s fluid an azure A at pH 7 0 and pH 2 0 Resolubilization of chondroitin sulphate occurred however when methanol fixed sections were immersed in water This was not observed when sections fixed in formalin containing cetylpyridinium chloride were immersed in 70 per cent ethanol or in absolute ethanol

It is to be expected that other acid glycosaminoglycans in tissues will behave by and large in the same way as chondroitin sulphate possibly with the exception of keratan sulphate which is soluble in aqueous solutions containing an excess of cetylpyridinium chloride

The autoradiographic investigation revealed inter alia that using the stripping film technique no distinct autoradiograms could be obtained on frozen sections stained in azure A This seemed to depend on alterations of the photographic emulsion caused by the stain

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ULTRASTRUCTURAL CHANGES IN RAT MAST CELLS DURING ANAPHYLACTIC HISTAMINE RELEASE

By

NIRMAL CHAKRAVARTY GUNNAR T GUSTAFSON
and ERIK PIHL

Received 19 ix 67

When a foreign protein together with *Pertussis* bacilli is injected into rats sensitizing antibodies develop in the body within a few days and cause sensitization of mast cells apparently as a result of fixation of the antibodies to these cells (*Mota* 1964)

The peritoneal cell suspension from rats offers a readily available source of sensitized mast cells which can be used *in vitro* If these cells are incubated with the sensitizing protein an anaphylaxis like phenomenon occurs *in vitro* and histamine is released to the suspending medium

The work on histamine release during the last few years leaves little doubt that histamine must be present in the mast cell granule in some form of ionic binding from which it is readily dissociable As proposed by *MacIntosh & Paton* (1949) such negatively charged groups for basic histamine could very well be provided by heparin which is a predominant constituent of the mast cell granule (*Holmgren & Wilander* 1937 *Hagen et al* 1959 *Parekh & Glick* 1962 *Lagunoff et al* 1964)

Recently an electron dense staining of mucopolysaccharides by Ruthenium red has been described Ruthenium red reacts conspicuously with the mast cell granule and the reaction seems to be specific for heparin (*Gustafson & Pihl* 1967) We have used here this staining method combined with electron microscopy to study the changes in the mast cells following brief incubation of the cells *in vitro* to induce histamine release by antigen antibody reaction and compound 48 80

MATERIAL AND METHODS

Male Wistar rats weighing 200– 0 g were used For the experiments with sensitized mast cells the rats were actively sensitized with egg albumin as described in a separate communication (*Chakravarty* 1967)

This work was supported by grants from the *Swedish Medical Research Council* (Project No 13X–53?–0?) and from the *Faculties of Odontology and Medicine*

The rats were anaesthetized lightly with ether and bled from the carotid arteries 8 ml of Krebs Ringer solution without calcium (143 mM NaCl 4 7 mM KCl 1 2 mM $MgSO_4$ 3 1 mM Na_2HPO_4 + KH_2PO_4 buffer pH 7 4) containing in addition 15 5 mM sodium citrate were injected into the peritoneal cavity of each rat The abdomen was gently massaged for 90 seconds and the mixed cell suspension containing mast cells was collected in a centrifuge tube Cells were usually pooled from 2–4 rats for each experiment as shown in Table 1 After centrifugation at 40 g for 6 minutes the cells were resuspended in a solution having the same composition as the one mentioned above except that sodium citrate was now omitted and the new solution contained in addition 2 5 mM $CaCl_2$ (replacing equimolar amount of NaCl) 5 mM glucose and 1 mg/ml human serum albumin (final pH 7 0–7 1) The cell suspension was divided into several samples and incubated under gentle shaking at 37 C. After an initial incubation for 5 minutes the antigen (egg albumin) or compound 48/80 (AB Leo Halsingborg Sweden) was added to some of the samples the others serving as controls and incubation was continued for another 5 minutes The samples were in duplicate one being used for testing histamine release and the other for electron microscopy The cells were kept at 0–4 C throughout the experiment except for the brief period of incubation at 37 C The glassware used for the cell suspension were silicone coated

Histamine release was determined as described elsewhere (*Chakravarty* 1967) using the biological testing method

For electron microscopy the specimens were treated as described earlier (*Gustafson & Pihl* 1967) After incubation with the histamine releaser the cells were chilled and suspended for 15 minutes in 4 ml 0 1 per cent Ruthenium red in 0 1 M phosphate or trismaleate buffer containing 7 5 per cent sucrose pH 7 4 temp 0–4 C The Ruthenium red stained cells were then washed in phosphate buffer and the centrifuged cell deposit fixed in 4 per cent phosphate buffered glutaraldehyde In some experiments cells were also postfixed in Millonig's phosphate buffered OsO_4 The cells were then dehydrated and embedded in Epon 812 (*Luft* 1961) The temperature of the specimens during all the steps to embedding was kept at 0–4 C Ultrathin sections were cut on LKB Ultramicrotomes and collected on unsupported copper grids Contrasting was generally avoided so as not to mask changes in electron density produced by Ruthenium red The sections were examined in a Siemens Elmiskop I A at 60 kV One micron thick sections were examined in the light microscope directly or after additional toluidine blue staining

RESULTS

Fig 1 shows a normal peritoneal mast cell stained with Ruthenium red Granules of different electron density are spread over the cell

Experiments with Compound 48/80

Figs 2–7 show the effect of compound 48/80 (0 1–0 2 µg/ml) which induced 18–51 per cent histamine release We have used the Ruthenium red technique in all electron micrographs except one (Fig 5) in which the conventional technique was used for comparison In gross sections

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Figs 1 and 2

Fig 1 Normal peritoneal mast cell There is a great difference in electron density between different granules Granules of different electron density are irregularly scattered × 21 000

Fig 2 This mast cell was exposed to compound 48/80 postfixed in OsO_4 and stained with uranyl acetate The cell shows localized disruption of the cell membrane exposing a granule to the extracellular environment × 48 000

All electron micrographs except Fig 5 were taken from specimens stained with Ruthenium red No postfixation in OsO_4 or additional staining was carried out if not otherwise stated

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Fig 1 Normal peritoneal mast cell There is a great difference in electron density *between different granules* Granules of different electron density are irregularly scattered × 21 000

Fig 2 This mast cell was exposed to compound 48/80 postfixed in OsO_4 and stained with uranyl acetate The cell shows localized disruption of the cell membrane exposing a granule to the extracellular environment × 43 000

All electron micrographs except Fig 5 were taken from specimens stained with Ruthenium red No postfixation in OsO_4 or additional staining was carried out if not otherwise stated

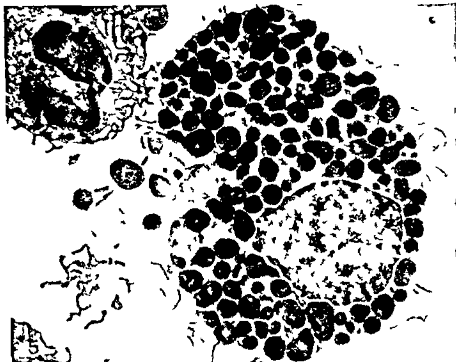


Fig 5

This cell was treated as described in Fig 2 but not stained with Ruthenium red The changes produced by compound 48/80 as shown by this technique consist of pale and enlarged granules around a localized disruption of the cell membrane × 8 800

of the material represented in Figs 2–7 varying degrees of mast cell changes were observed Figs 2–5 demonstrate relatively mild changes confined to a part of the cell A localized disruption of the membranes with a granule apparently in the process of being released is shown in Fig 2 Cytoplasmic changes with a clear space is seen at the side of the granule Fig 3 shows a vesicle protruding from the cell surface the form and size of which suggest that this structure is related to a released granule Cytoplasmic changes and granules having fuzzy outlines and without surrounding membranes may be seen adjacent to the vesicle Some granules have intact membranes but membrane fragments without relation to granules may be seen Some of the changes described above are also seen in Fig 4

Figs 3 and 4

These cells were incubated with compound 48/80 Postfixed in OsO_4 Varying degrees of localized changes consisting of [illegible] halos bulging of the cell membrane and disruption of perigranular membranes can be seen The "bubble" in Fig 3 may represent perigranular and cell membrane rests after extrusion of a granule × 30 000 × 4 000

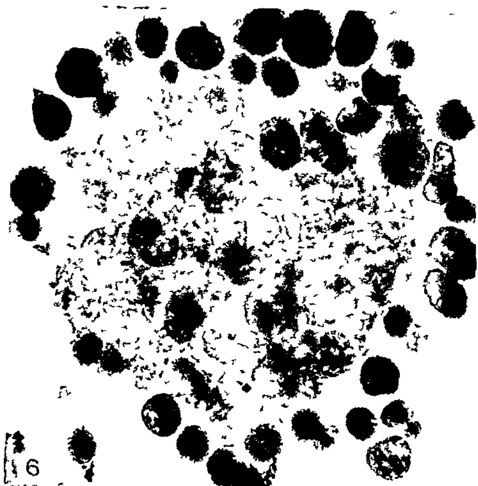


Fig 6

This mast cell demonstrates a generalized change caused by compound 48 80 The central granules appear swollen and very little cytoplasm is seen in between The peripheral granules are highly electron dense and generally smaller than the central ones × 22 000

Fig 5 in which no Ruthenium red was used shows localized disruption of the cell membrane and release of a few granules The latter show reduced electron density in contrast to the general high electron density of the peripheral granules after Ruthenium red staining the high electron density of such granules is seen more clearly in Fig 6 The centrally situated granules increased in size in comparison to the more electron dense peripheral granules In more severely affected cells (Fig 7) Ruthenium red positive material had leaked out and the granule structure partly disintegrated

The mast cell changes described above were not seen in control experiments without compound 48 80

Experiments with Antigen

When unsensitized cells were incubated with the antigen (0 5 mg ml egg albumin) their appearance did not differ from that of untreated controls (Fig 1)

TABLE 1

Histamine Release from Rat Peritoneal Mast Cells by Compound 48/80 and Antigen Antibody Reaction Duplicate Samples Were Used for Electron Microscopy Cells Were Pooled from 2–4 Rats for each Experiment

Expt		Conc of releaser	Histamine release (%)
1	Compd 48/80 (µg/ml)	0 1	27
		0 2	37
2		0 1	18
		0 2	51
3	Antigen egg albumin (mg/ml)	0 5	40
4		0 5	14
5		0 1	34
6		0 1	31

The spontaneous histamine release in the controls was 0–3 per cent
The release with egg albumin from unsensitized mast cells was within the same range

Incubation of sensitized cells with antigen gave 14–40 per cent histamine release (Table 1) and caused essentially the same kind of mast cell changes as observed after exposure to compound 48/80 Fig 8 shows central large and pale granules surrounded by highly electron dense peripheral granules Fig 9 shows a mast cell with more marked changes In spite of the disruption of perigranular and cell membranes the granules in general retained their shape homogeneity and outline

DISCUSSION

The anaphylactic changes in mast cells as judged by comparison with control material consisted of an alteration in distribution size and electron density of the granules Essentially the same pattern of structural changes in the mast cells was observed in all the experiments in which histamine was released by compound 48/80 or antigen The changes were often localized to a small part of the cell with release of a few granules In other cases in which the entire cell was affected centrally situated granules were large pale and often surrounded by relatively smaller and highly electron dense peripheral granules The large size of the central granule seems to be due to swelling because they usually formed together a compact mass with little cytoplasm in between (Fig 6) The swelling could conceivably be produced by a change in the permeability of the perigranular membrane at a stage when no membrane disruption may be detected in the electron microscope Recent works by *Lagunoff et al* (1964) *Uvnas* (1964) and *Thon & Uvnas* (1966) with isolated granules suggest that the selective

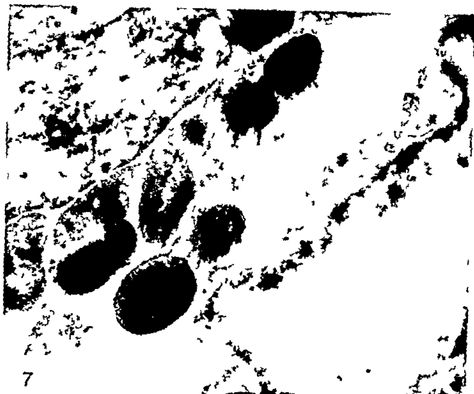


Fig 7

Cells incubated with compound 48/80 Between two nuclear rests a few mast cell granules are seen Their structure is apparently disintegrating Electron dense Ruthenium red positive material is seeping out of the granules × 23 000

permeability of the perigranular membrane to ions may be a determining factor in maintaining the high concentration of histamine within the granules A change in the permeability of the granule membrane could then result in an inflow of ions and water causing granular swelling

Assuming that the swelling of the central granules is caused by an entry of water and ions the inflow of cations into the granule could lead to some histamine release into the cytoplasm at this stage Such peripheral granules which had come in contact with the extracellular milieu would more easily release histamine to extracellular fluid by an ion exchange mechanism This is suggested by the observation that isolated granules—which apparently have no membranes (*Lagunoff et al* 1964)— retain most of their histamine in distilled water (*Uvnas* 1964) and sucrose solution (*Lagunoff et al* 1964) but lose it rapidly in salt solutions (NaCl KCl or CaCl)

The reason why the peripheral granules in Figs 6 and 8 are comparatively small and more electron dense is not clear One might speculate that with the disappearance of the membrane the granule incurs a

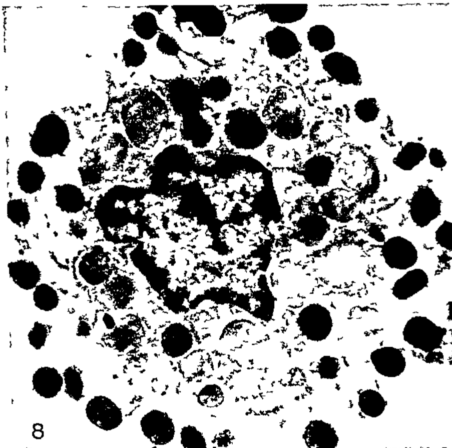


Fig. 8

Sensitized mast cell following incubation with 0.1 mg/ml antigen. The nucleus looks rather normal. The central granules are larger and paler than those at the periphery which are very electron dense. Thus the general appearance of the cell is similar to that obtained after incubation with compound 48/80 (compare Fig. 6). × 20 000

loss of small molecular weight components accounting for the shrinkage in size and high electron density. It may also be that the contact of these granules with the extracellular fluid permits more effective fixation and staining and possibly also some shrinkage.

The morphological changes described above were similar following antigen antibody reaction and application of compound 48/80. This is consistent with the view adopted by *Chakravarty* (1959) and *Mota & Ishii* (1960) that very similar mechanisms are involved in anaphylactic and compound 48/80 induced histamine release in rats.

Electron microscopic studies of mast cells after exposure to compound 48/80 *in vitro* (*Bloom & Haegermark* 1965) and *in vivo* (*Singleton & Clark* 1965) have recently been reported but as far as we are

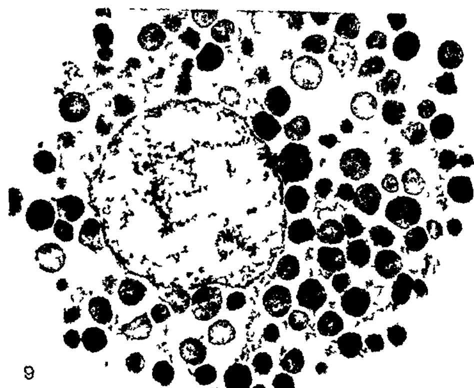


Fig 9

Mast cell incubated with antigen Here the cytoplasmic changes are more severe than those seen in Fig 8 In addition there is a breakdown of nuclear structure At this advanced stage differences in granule electron density and size are less pronounced as compared to those seen in Fig 8 × 13 000

aware no studies have been published on antigen induced changes in the mast cells simulating an anaphylactic reaction *in vitro* The effect of rabbit anti rat gamma globulin on the mast cell has been studied by *Keller* (1966) This reaction however is of a different nature and requires complement (*Austen & Bloch* 1965) *Bloom & Haegermark* (1965) and *Singleton & Clark* (1965) have reported that compound 48/80 causes the appearance of large pale granules with clear halos referred to as altered granules This type of change—although less pronounced—has been seen in our material (Figs 3 4 and 6) This difference seems to stem from the different techniques used and will be further investigated

In addition the use of Ruthenium red permits us to follow the fate of heparin by electron microscopy after the cells have been exposed to the histamine releasing agents Heparin is known to be released in anaphylactic reaction in dog (*Wilander* 1938 *Jaques & Waters* 1941) but no heparin release has been shown to accompany anaphylactic histamine release in other species (*Adams* 1953) This discrepancy may at least partly depend on the relative ease with which the released

heparin can reach the bloodstream in dog compared to other species (*MacIntosh* 1956 *Riley* 1956) Although *Archer* (1961) did not find any evidence of heparin release by a single application of compound 48/80 to rat mast cells *Riley et al* (1955) could produce a 50 per cent reduction of the heparin content of rat connective tissue by repeated doses of compound 48/80 which caused practically complete depletion of histamine Our electron micrographs show that while heparin may stay in the granules of cells with characteristic changes compatible with histamine release more severe damage to the cells leads to leakage of heparin from the granules (Fig 7)

SUMMARY

The ultrastructural changes in rat peritoneal mast cells accompanying histamine release were studied using Ruthenium red which reacts specifically with the acid mucopolysaccharides (glycosaminoglycans) of the mast cell granules Antigen applied to actively sensitized cells and compound 48/80 were used as histamine releasing agents

Changes in granule size electron density and distribution produced by antigen and compound 48/80 were observed Milder changes were associated with localized release of granules The severely affected cells usually showed swollen central granules while the peripheral granules in contact with extracellular fluid were generally smaller and electron dense

The changes observed seem to reflect essential events in the anaphylactic reaction leading to histamine release

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HAEMORRHAGIC NEPHRITIS IN EXPERIMENTAL BOVINE LEPTOSPIROSIS *BRATISLAVA*

By

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Received 22 iv 67

The pathological manifestations of leptospiral infection are generally believed ultimately to be due to toxic substances released from the leptospires in the infected organism

Apart from pyrogenic cell wall constituents similar to the endotoxins of other gram negative organisms (22) parasitic leptospires may produce distinct substances that condition the occurrence of distinct clinical forms of leptospirosis icteric haemorrhagic haemoglobinaemic etc (13) however the exact mechanism of leptospiral pathogenicity is largely unknown

The classification of leptospires in serotypes owes its practical value in part to the correlation often existing between the pathogenic properties and the agglutinogenic constitution of newly isolated leptospira strains

The serological type *bratislava* syn *erinacei europaei* syn *esposito* was differentiated from other *Leptospira* serotypes independently by *Kmety* (14) *Ananyin* (1) and *Smith & Brown* (23) Cross absorption tests (2 9 16 17 25) have shown that the various designations proposed for this new serotype are actually synonyms

In Europe the maintaining host for leptospires of the *bratislava* serotype appears to be the European hedgehog *Erinaceus europaeus* (1 3 6 9 12 14 15 19 20 21 24 26)

The pathogenicity of newly isolated *bratislava* leptospires for weanling hamsters is high infected animals usually dying within 8 days with pronounced haemorrhagic lesions but without jaundice The pathogenicity for weanling guinea pigs is somewhat lower a varying proportion of the infected animals die with pronounced haemorrhagic

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TABLE 1

History of bratislava Strains Used for Production of Experimental Leptospirosis in Calves with Details of the Inoculations

Experiment no	Strain	Date of isolation	Maintenance of strains from day of isolation until day of inoculation	Inoculation of calves§	Age and No of calves			Approx number of guinea pig infective doses
					1 to 3 days	19 to 23 days	34 to 40 days	
1	P 13	June 29 1959	*in vitro* 7 days 2 hamster passages mouse 237 days and 6 guinea pigs passages	April 7 1960		31 32		10
2	P 22	July 21 1959	*in vitro* 30 days mouse 201 days 3 guinea pig passages *in vitro* 13 days and 13 guinea pig passages	June 11 1960	1 2	33 34	51 52†	10^{3}
3	P 156	June 20 1961	*in vitro* 31 days and 5 guinea pig passages	Aug 14 1961		35 36	53 54	10^{3}
4	P 156	June 20 1961	as above plus 11 guinea pig passages	Oct 5 1961	3 4		55 56	10
5	P 156	June 20 1961	as above plus 1 calf and 2 guinea pig passages	Oct 27 1961	5 6		57	10^{4}

All strains were isolated in Denmark from *Erinaceus europaeus*

§ Calves were inoculated subcutaneously with 1 ml 10 0 per cent guinea pig liver suspension

† This calf which died from traumatic pericarditis on the 19th p i day is not included in the final material

lesions quite often involving the kidneys and not rarely accompanied by jaundice. Weanling mice usually survive *bratislava* infection without notable symptoms but often become leptospira carriers (4, 8, 26).

Human *bratislava* infections are usually mild and anicteric (14, 23 and personal communications from *Kmety* and *Ananyin*).

Clinical disease caused by *bratislava* leptospires in domestic animals has not been reported but serological surveys have provided evidence that *bratislava* infection does occur in cattle, swine, horses and dogs (7, 18, 20, 21, 24).

The present paper reports the results of experiments conducted in order to investigate the pathogenicity of *bratislava* leptospires for cattle. Young calves were chosen as experimental animals since it has been observed that leptospires manifest their potential pathogenicity more readily in immature than in mature cattle (11).

MATERIALS AND METHODS

Experimental Animals

Nineteen apparently healthy calves of the Red Danish Dairy Cattle breed (R.D.M.) were inoculated but one was later excluded from the material because of intercurrent disease (see Table 1). All calves were males except two (Nos. [illegible] and 32). During the observation period they were fed with milk supplemented by hay or grass. At the time of inoculation their serum did not contain agglutinins demonstrable in dilution 1:10 or higher against leptospires of the following serotypes: *icterohaemorrhagiae*, *poi*, *canicola*, *ballum*, *bratislava*, *pomona*, *grippotyphosa*, *sejroe*, *saxkoebing*, *bataviae* and *hyos* with the exception of the serum from one newborn calf (No. 3) which had a *saxkoebing* titre of 10. Restricted facilities prevented simultaneous inoculation of more than a few calves.

Leptospira Strains

The history of the three *bratislava* strains used and details of the inoculations are given in Table 1. The pathogenicity of strain P 156 for guinea pigs was somewhat less than that of strains P 13 and P [illegible], the lethality being 3[illegible] and 48 per cent respectively.

Observation of Inoculated Calves

The temperature of the calves [illegible]s taken at least twice a day and since a rise in temperature was always the first sign of disease to be observed the first fever day was considered the first day of disease. During and occasionally also before and after the febrile stage blood was examined for leptospires by guinea pig inoculation. Serum was examined daily for leptospira agglutinins in dilution 1:10 until positive and thereafter twice a week. Examinations for blood urea and haemoglobin were performed every second or third day during the first tw[illegible] weeks of disease, thereafter less frequently. Freshly voided urine w[illegible]s [illegible]xam[illegible]n[illegible]d every second or third day for protein by Albustix reagent strips and f[illegible] leptos[illegible] [illegible] b[illegible] dark field microscopy.

Calves that died were exam[illegible]n[illegible]d for l[illegible] [illegible]p[illegible]es in blood, liver and kidneys by cultivati[illegible]n in Korthof's medium and by g[illegible] [illegible] pig inoculation and for other bacteria by aerobic cultivation on blood aga[illegible] [illegible] [illegible]ns were rec[illegible]rded and samples from liver, kidney, spleen and lungs wer[illegible] [illegible]x[illegible]mi[illegible]ed histologically.

Calves that survived the a[illegible]ut[illegible] [illegible]e[illegible]t[illegible]n were slaughtered wh[illegible]n lept[illegible]piruria apparently had terminated or in [illegible]n[illegible] [illegible] without detectable leptospiruria when the agglutinin titre was declining. Th[illegible] were examin[illegible] [illegible] [illegible]r[illegible] [illegible]ions and their kidneys [illegible]ere examined hist[illegible] [illegible] [illegible] [illegible]nd for lep[illegible] [illegible] [illegible] [illegible] [illegible]tion and guinea pig inoculation.

TABLE 2

Incubation Period and Clinical Response in Calves Infected with bratislava *Leptospires*

Group	Calf no	Incubation period (in days)	Fever (max °C)	Anorexia	Maximum degree of depression: severe	Maximum degree of depression: moderate	Haemorrhagic faeces	Haematuria	Uraemia (max blood urea mg%)	Proteinuria (max degree)	Death
A 1	1	5	1-3 (40 7)	3-6	2-6		2-6	§	3-6 (?64)	?-6 (+++)	6
	33	3	1-5 (41 0)	5-8	5-8		6-8	6-8	5 8 (?93)	4-8 (+++)	8
	3	5	1-3 (40 7)	4-14	4-14		6-13	5-12	3-14 (613)	3-14 (+++)	14
A 2	31	4	1-4 (41 1)	4-6	4-6			4-11	4-83 (?96)	3-83 (+++)	
	31	4	1-7 (41 ?)	4-7	5-6			7-13	4-67 (265)	3-51 (+++)	
	2	5	1-3 (40 2)	3-4	3		5-7	3-5	3-47 (140)	2-31 (+++)	
	35	5	1-4 (40 9)			2-7	5-7	4-8†	4-8 (149)	4-15 (++)	
	4	6	1-5 (40 5)			?-4		6-7†	5 (63)	4-10 (+)	
B	5	5	1-5 (41 0)		3 4				(35)	4-13 (+)	
	6	6	1-3 (41 1)		2-3				3 (49)	3-21 (+)	
	3?	4	1-7 (41 3)			3-5			(41)	4-9 (±)	
	36	4	1-7 (42 4)			2-7			7-25 (57)	5-9 (±)	
C	51	3	1-6 (41 4)						(42)		
	53	4	1-6 (41 6)						(35)		
	54	4	1 6 (40 7)						(41)		
	55	4	1-4 (41 5)						(33)	5 (±)	
	56	4	1-4 (41 1)						(33)		
	57	5	1-4 (40 9)						3 (47)		

Figures refer to days of disease unless otherwise stated § Anuria from day 3

† Demonstrable only in urine sediment

Microscopical Cultural Guinea Pig Inoculation Serological and Histological Methods These were similar to those described previously (11) except that the tissue sections were also stained by PAS haematoxylin Unna Pappenheim Masson s trichrome 0 5 per cent aqueous toluidine blue and Astra blue (5)

RESULTS

Clinical Observations

A survey of the most important symptoms and signs is given in Table 2 in which the calves are grouped according to the severity of their acute clinical disease (Groups A B and C see later)

After an incubation period of 3 to 6 days slightly longer in newborn than in older calves (average 5 3 and 4 0 days) all the animals developed a febrile reaction which lasted for 3 to 7 days being of slightly shorter duration in newborn than in older calves (average 3 7 and 5 3 days) Maximum temperatures which ranged from 40 2 to 42 4 C were slightly lower in newborn than in older calves (average 40 7 and 41 3 C) Actual fever relapses did not occur but slight ephemeral fever attacks were noted in five calves (Nos 31 32 36 53 57) during the 2nd to the 6th weeks of disease

All calves showed slightly reduced activity and decreased interest in their surroundings during the febrile period Moderate depression in the form of definite dullness and a tendency to remain recumbent was observed in all the newborn and 3 week old calves beginning on the 2nd to 4th day of disease This condition developed rapidly into severe depression characterized by complete lack of interest in their surroundings and unwillingness to rise and usually also by anorexia in five newborn and three 3 week old calves The severe depression continued in three animals until they died in the other calves it subsided gradually within a few days

Conjunctival congestion was observed in almost all the calves usually commencing about the 5th day and lasting only for a few days but in one calf (No 31) persisting for more than 3 weeks A transient serous ocular discharge was noted in four calves during the 1st and 2nd weeks

Irregular spells of diarrhoea and constipation occurred in most of the animals particularly during the 1st and 2nd weeks

Group A A haemorrhagic nephritic syndrome with various degrees of haematuria (or in one case anuria) proteinuria and uraemia was observed in four newborn and four 3 week old calves Three of these animals died during the acute phase of disease (Group A 1) while five survived (Group A 2)

Haematuria began at the time of fever defervescence and lasted for 2 to 8 days In two calves it was slight being visible only after sedimentation In the other five animals the freshly voided urine was opaque red or pink being quite different from the translucent red coloured urine characteristic of haemoglobinuria There were no blood

TABLE 3

Blood Urea in Calves Infected with bratislava Leptospires

Clinical group	Calf no	Week of disease: 1st				2nd			3rd to 6th				7th and later			
A 1	1		101^{3}	194^{5}	264^{6}†											
	33	25		113^{5}	207^{7}	293^{8}†										
	3	20^{1}	70^{3}	156^{5}	261^{7}	355^{9}	479^{1}	*613^{14}*†								
A 2	34	24^{1}	138^{4}	245^{6}	*$29?^{7}$*	275^{9}		193^{13}	125^{16}	84^{5}	101^{31}	66^{41}	91^{48}	€2	43^{6}	f6^{88}
	31	20	51^{4}	164^{6}	265^{7}	264^{10}	202^{12}	154^{14}	125^{16}	109^{3}	80^{32}	83^{38}	67^{44}	44^{55}	52^{67}	
	2		66^{3}	*140^{3}*	$1?5^{6}$	69^{8}	56^{12}		64^{15}	47^{7}	51^{33}	52^{40}	50^{47}	35^{54}	31^{68}	30^{95}
	35	32	101^{4}	*149^{6}*		67^{8}	42^{10}	38^{1}		34^{4}	38^{31}	23^{38}	11^{45}			
	4	27^{1}	29^{3}	63^{4}		30^{9}		24^{13}	28^{16}	26^{3}	22^{30}	25^{37}				
B	5	26^{1}	32^{3}		*35^{6}*	29^{8}	21^{11}	26^{13}	28^{16}							
	6	18^{1}	*49^{3}*	33^{5}	36^{7}		26^{12}		27^{15}							
	32	22^{2}	25^{4}	39^{6}	37^{7}	*41^{10}*	27^{1}	24^{14}	23^{16}	*29^{3}*	26^{9}		25^{44}			
	36	36^{1}	34^{3}	28^{5}	57^{7}	46^{9}	*51^{11}*			46^{5}	26^{32}					
C	51	25^{2}		26^{5}	36^{7}	25^{8}	28^{10}		36^{14}	34^{21}	42^{8}	34^{35}	29^{43}			
	53	28^{1}	26^{3}	35^{5}	24^{7}	25^{9}	29^{11}	30^{13}		23^{5}	*21^{3}*	23^{39}				
	54	31^{1}	29^{3}	37^{5}	24^{7}	27^{9}	33^{11}	*41^{14}*		16^{5}	27^{32}	21^{39}	17^{46}			
	55	26^{1}	*33^{3}*	27^{6}		19^{9}	20^{10}		18^{15}	15^{5}	8^{3}	17^{39}				
	56	22^{1}	*33^{3}*	26^{6}		21^{8}	19^{10}			13^{5}	11^{32}	15^{39}				
	57	35^{1}	*47^{3}*	35^{6}		34^{8}	33^{11}		23^{16}							

Exponential figures indicate day of disease. Italic figures indicate peak values observed. † Day of death

clots to indicate post renal haemorrhage Erythrocytes were not seen in the few drops of urine found in the bladder at *post mortem* examination of Calf 1 which had shown anuria from the 3rd day of disease

The diuresis of the animals was not measured but in addition to anuria in Calf 1 pronounced oliguria was noted in Calves 3 4 and 31 for one or two days during the latter half of the first week

Proteinuria usually began on the 3rd or 4th day *i e* one to several days before haematuria became detectable and reached its peak 1 to 7 days later It was then marked (+ + + corresponding to 300 mg% or more) in the calves with anuria or gross haematuria and less pronounced in those with slight haematuria The proteinuria decreased gradually in the five surviving calves and ceased in four of them during the 2nd 3rd 5th and 8th weeks but there were still traces of protein in the urine of Calf 34 when this animal was sacrificed 12 weeks after the onset of disease

Blood urea values above 45 mg% were usually first observed on the last day of fever (see Tables 2 and 3) Maximum values between 264 and 613 mg% were reached in the terminal stage of illness in the three fatal cases In Calves 31 and 34 which had shown maximum values of 265 and 296 mg% on the 7th day the blood urea values decreased only slowly and were still slightly above 45 mg% when these animals were sacrificed In Calf 2 which had shown a maximum value of 140 mg% on the 5th day normal values were reached in the 8th week In Calves 4 and 35 normal values were reached already in the 2nd week of disease

Hyaline granular epithelial leucocytic and erythrocytic casts were seen frequently in the urine of Calves 2 31 and 34 during the 2nd to the 4th weeks and occasionally in the urine of Calf 35 during the 2nd week

Blood coloured faeces were observed in five calves beginning on the 2nd to the 6th day The blood admixture consisted initially of streaks of fresh blood and small clots this condition persisted for 3 days in two animals whereas the amount of blood increased gradually in the fatal cases

Clinical signs of anaemia or intra[illegible] [illegible]aemolysis were not noted but a moderate decrease in haem[illegible]bin [illegible]ntration (2 2 to 3 4 g%) was found in the three calves [illegible]at died

Jaundice was not observed [illegible] calves but s[illegible]ht jaundice was noted at the *post mortem* examination of Calf 34

A marked loss of hair [illegible]urred on the lateral surfaces of the extremities the abdomen th[illegible] base of the [illegible]ars and the surroundings of the eyes of Calf 34 during th[illegible] 3rd to the 5th week

Group B A moderate clinical response characterized by fever and severe or moderate depression but no haematuria or anuria was seen in two newborn and two 3 week old calves Slight proteinuria of 1 to

In Calf 33 which died on the 8th day the proliferating mesenchymal cells contained pyroninophilic substance in the cytoplasm (Fig 1 A) and the ground substance showed strong metachromasia after staining with toluidine blue (Fig 1 B) and a strong blue colour after Astra blue staining (Fig 1 C) indicating the presence of acid mucopolysaccharides The van Gieson Hansen stained sections showed beginning differentiation of collagen fibres Scattered leucocytic casts were noted in some tubules in this calf Some small foci of lymphocytes and plasma cells were present in the cortex

In Calf 3 which died on the 14th day the mesenchymal cells showed pyroninophilia though slightly weaker than in Calf 33 The content of acid mucopolysaccharides in the ground substance was lower than in Calf 33 as judged by the less intense metachromasia and Astra blue staining The van Gieson Hansen stained sections showed a vivid formation of collagen fibres (Fig 1 D) Some foci of lymphocytes and plasma cells were noted this infiltration being slightly more pronounced than in Calf 33 Some tubules had undergone progressive atrophia and this was most pronounced in Calf 3

Histological examination of liver and spleen from the three calves revealed severe hyperaemia but otherwise there were no significant lesions and particularly no signs of hepatitis or liver cell necrosis

Group A 2 In Calves 31 and 34 which were sacrificed 2 and 3 months after the onset of severe disease the kidneys appeared to be slightly smaller than normal and the texture was increased considerably Close examination of the decapsulated surface revealed that the parenchyma was divided into numerous tiny islets separated by thin greyish strands giving the surface a faintly net like appearance In addition several pin head sized greyish white foci were seen in Calf 34, and many scattered irregularly shaped greyish areas measuring 0 5 to 5 mm in width in *Calf 31* On the cut surface the cortex appeared to be contracted and greyish streaks were seen to extend from the capsular surface towards the medulla in the kidney of Calf 31

Microscopically there were focal infiltrations with lymphocytes and plasma cells often surrounding the arterioles in the renal cortex of all five calves (*Figs 4 and 5*) However the outstanding finding in

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Fig 1

Development of the interstitial renal fibrosis in calves experimentally infected with *bratislava* leptospires

A Calf 33 died on the 8th day of disease Intense proliferation of primitive mesenchymal cells containing large amounts of pyroninophilic substance in the cytoplasm (Unna Pappenheim)

B Same calf Accumulation of metachromatic intercellular substance (Toluidine blue 0 1 per cent)

C. Same calf The intercellular substance is positive to Astra blue (Astra blue 0 1 per cent in 1 per cent acetic acid counterstaining with kernechtrot)

D Calf 31 sacrificed 67 days after the onset of disease Massive interstitial fibrosis atrophy of tubules (van Gieson Hansen)



Fig 1

TABLE 5

Bacteriological and Serological Findings in Calves Infected with bratislava *Leptospires*

Clinical group	Calf no	Leptospiruria (duration in days)	Leptospires in kidneys (day)	Agglutinins first demon on day	Period of rising titres (in days)	Max titre (attained on day)	Titre at death or sacrifice (day)
A 1	1	none	+ (6)	6	—	10 (6)	10 (6)†
	33	none	+ (8)§	8	—	30 (8)	30 (8)†
	3	none	+ (14)	8	—	300 (12)	300 (14)†
A 2	34	21–30 (10)	0 (84)	6	10	30 000 (16)	300 (84)
	31	14–38 (25)	0 (67)	7	13	3 000 (20)	3 000 (67)
	[illegible]	26–36 (11)	0 (83)	5	22	3 000 (27)	3 000 (83)
	35	10–51 (42)	+ (62)	6	11	3 000 (17)	1 000 (62)
	4	[illegible] (44)	0 (70)	6	45	30 000 (51)	30 000 (70)
B	5	8–49 (41)	+ (70)	9	42	10 000 (51)	10 000 (70)
	6	10–47 (38)	+ (69)	9	41	3 000 (50)	3 000 (69)
	3[illegible]	11–60 (50)	0 (67)	6	12	10 000 (18)	10 000 (67)
	36	11–34 (24)	+ (54)	7	6	3 000 (13)	3 000 (54)
C	51	none	0 (57)	7	5	1 000 (12)	300 (57)
	53	14–23 (10)	0 (54)	7	4	3 000 (11)	1 000 (54)
	54	11–43 (33)	0 (63)	7	9	3 000 (16)	1 000 (63)
	55	9–31 (23)	0 (46)	6	9	10 000 (15)	3 000 (46)
	56	10–31 (22)	0 (46)	6	9	3 000 (15)	1 000 (46)
	57	9–29 (21)	0 (49)	6	10	3 000 (16)	3 000 (49)

Figures refer to day of disease unless otherwise stated

§ Lept[illegible] from peritoneal fluid liver and blood

† The calf died

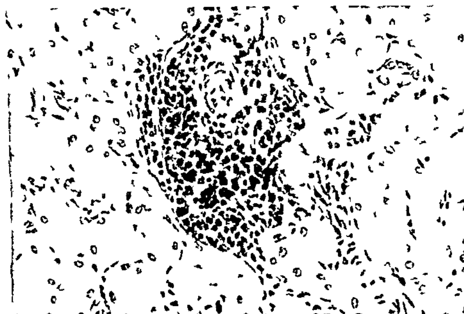


Fig 5

Same calf as Fig 4 Focal infiltration by plasma cells and lymphocytes (Unna Pappenheim)

Bacteriological and Serological Findings

Table 5 summarizes the bacteriological and serological findings Leptospiraemia was not investigated systematically but leptospires were found in the blood of all the calves during the febrile stage and in some cases as early as two days before the onset of fever

Leptospires were isolated from blood and peritoneal fluid (and from liver and kidneys) of Calf 33 which died on the 8th day with a *bratislava* agglutinin titre of 30 but only from the kidneys of Calves 1 and 3 which died on the 6th and 14th days with *bratislava* titres of 10 and 300 respectively

Leptospiruria was demonstrated in all the surviving calves except one In most animals leptospires became detectable in the urine during the 2nd week but in two animals not until the 3rd or 4th week of disease The microscopically detectable leptospiruria lasted for 10 to 50 days however in four calves leptospires were cultured from the kidneys 11 to 22 days after the latest microscopic detection of leptospiruria (see Table 5) The leptospires seen in the urine were often poorly preserved more or less agglutinated or located within mucous masses

Leptospira agglutinins appeared in the blood on the 5th to the 9th day of disease Maximum titres of 1 000 to 30 000 were reached within one to two weeks in 3 week and 5 week-old calves but not until three

calves Moreover severe renal changes and leptospiruria of more than five weeks duration were seen only in calves of the two former age groups

Strain P 156 appeared to be less pathogenic than strains P 13 and P 22 but was nonetheless able to cause severe disease

The longer periods of incubation shorter periods of fever lower maximum temperatures and longer periods of rising agglutinin titres in newborn calves may reflect a less effective defence mechanism However in the older calves there was no correlation between the above parameters and the course of disease

The *haemorrhagic nephritic syndrome* with haematuria but without haemoglobinuria in bovine leptospirosis has not been reported previously The same applies to the marked proliferation of primitive mesenchymal cells and the early formation of collagen fibres in the renal cortex of the calves that died and to the pronounced cortical fibrosis in two of the surviving animals

TABLE 7

Findings in Renal Cortex of Calves Infected with bratislava *Leptospires*

Histochemical findings in interstitial tissue	Interpretation	Death on day of disease		
		6th	*8th*	*14th*
Metachromasia and Astra blue staining of ground substance	Acid mucopolysaccharides	0	+++	++
Pyroninophilia and basophilia of cytoplasm of proliferating primitive mesenchymal cells	Nucleoproteins	0	+++	++
Fibres stained red with v Gieson and blue with Azan	Formation of collagen fibres	0	+	+++

The accumulation of acid mucopolysaccharides in the ground substance is presumably a precursory stage to the synthesis of collagen fibres and the large amounts of nucleoprotein in the primitive mesenchymal cells probably reflect the intense protein synthesis required for collagen formation This process resembles in many respects the formation of connective tissue in wound healing *Dunphy & Udupa* (10) who investigated the healing of wounds in rats by chemical and histochemical techniques described a normal pattern consisting of two phases a productive or substrate phase beginning 12 to 24 hours after wounding and lasting for about five days during which mucopolysaccharides and soluble protein precursors of collagen are produced presumably by fibroblasts and a collagen phase beginning about the 5th day and lasting until the completion of healing in which normal collagen fibres are formed From the histochemical findings

summarized in Table 7 it would appear that repair of the damage caused by *bratislava* leptospires in the bovine kidney follows a similar pattern The histochemical and clinical data indicate that the injury inducing the healing process occurred about the 3rd or 4th day of disease The marked renal fibrosis found in two of the surviving calves may be considered a late sequela of this process

The peculiar clinical and histopathological response of calves aged three weeks or less to infection with *bratislava* leptospires seems to indicate that the toxins of these leptospires are different from those present in leptospires of the serotypes *pomona* *grippotyphosa* and *sejroe* investigated under similar experimental conditions (11)

SUMMARY

The potential pathogenicity of serotype *bratislava* leptospires for cattle was investigated by infection of 18 young calves

A severe haemorrhagic nephritic syndrome with haematuria but without haemoglobinuria occurred in four newborn and four 3 week old calves Three of these animals died during the acute phase of the disease and two of the surviving calves had proteinuria and uraemia for many weeks The clinical response was moderate in two newborn and two 3 week old calves and mild in six 5 week old calves

The principal patho anatomical findings in the three calves that died were petecchial haemorrhage in various organs and tissues and en larged oedematous kidneys Massive proliferation of primitive mesen chymal cells in the renal cortex with early formation of collagen fibres and progressive tubular atrophy were the outstanding histo logical features

Patho anatomical findings in the surviving calves which were slaugh tered 6 to 12 weeks after the onset of disease were confined to the kidneys Small focal infiltrations with lymphocytes and plasma cells were found in the renal cortex of all calves except one Pronounced diffuse and focal renal fibrosis was seen in the two calves that had shown protracted proteinuria and uraemia

The distinctive severe clinical and histopathological response indi cates that the toxins of *bratislava* leptospires differ from those present in leptospires of the sero[illegible]pes *p*[illegible] *na* *grippotyphosa* and *sejroe*

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STUDIES ON THE COMPLEMENT FIXATION TEST WITH MYCOPLASMA PNEUMONIAE ANTIGEN

2 *Application of the Test to Hospitalized Pneumonia Patients and to Healthy Blood Donors*

By

JAN ENG

Received 29 iii 67

Many studies from different countries have shown that *M pneumoniae* is an important cause of pneumonia and that it can also give rise to other types of respiratory tract infection (2) The object of the present investigation has been to examine the prevalence of *M pneumoniae* infections in a group of Norwegian hospitalized pneumonia patients chiefly from the Oslo area using the complement fixation test (CFT) The same technique was also used in a series of sera from healthy blood donors

METHODS

The production of CF antigen and the CFT technique employed are described in a previous article (8) The sera we e screened in a dilution of 1 10 and sera showing 75 per cent or more inhibition of haemolysis were titrated using a geometrical dilution series The titre of a positive serum was the highest dilution of serum (before the addition of antigen and complement) giving 75 per cent or more inh bi tion of haemolysis

MATERIAL

1 *Cases of Pneumonia*

These include all the available frozen sera from patients with a clinical diagnosis f pneumonia sent to this laborato [illegible] from the clinic l departments of Oslo City Hospitals for serological virus investigation [illegible] nd o [illegible] cold agglutination test In the period 1959–1964 38 such sera were avail bl [illegible] f [illegible] m [illegible] 3 patients These were examined using antigen extracted with ether and f ndi gs [illegible] b en published pre iously (9) but they will also be mentioned briefly [illegible] th [illegible] nt [illegible] per During the period from June 1964 to June 1966 a furthe [illegible] 09 [illegible] ra [illegible] m [illegible] ti nts with pneumonia were recei ed These sera form the m t r l [illegible] th [illegible] n [illegible] tu [illegible] and ha e been examined with boiled antigen as described i [illegible] (3) 99 [illegible] f the e p [illegible] nts pro ided 1 sample 40 provided 2 samples and 6 provided 3 [illegible] mple [illegible] 4 [illegible] m [illegible] 60 patients were child en under 2 years old samples fr m these chil [illegible] r [illegible] ent in by the Paediatric Department Ulle ål Hospital [illegible] of th m [illegible] cluded in an in estig tion on respiratory syncytial viru [illegible] tions in [illegible] nf nts [illegible] blı hed by Ulstrup (23)

2 *Blo d Donors*

These sera from health [illegible] b [illegible] d donor [illegible] we e sent [illegible] by The Bloo l Bank Ullevål Hosp ital for routine ser l gi al testing f r syph lis n connecti n with every lona tion of blo d From July 19[illegible] to Januar [illegible] 1967 all such s [illegible] a [illegible] ere frozen 63 of these

sera from the same number of blood donors belonging in different age groups and of both sexes and all with a second sample taken 3 months after the first one were examined with boiled antigen as described in (8). When sample No. 1 showed a positive *M. pneumoniae* CFT (titre ≧ 1:10) sample No. 2 was also examined.

TABLE 1

Results of M. pneumoniae CFT in Sera from 151 Hospitalized Patients with Pneumonia from the Oslo Area in the Period 1964–1966

Age in years	Number of patients	Mycoplasma pneumoniae complement fixation test							
		Titre in 1 sample or in several samples with equal titres							Significant rise in titre
		Neg	1:10	1:20	1:40	1:80	1:160	1:320	
0 – 2	60	57	3	–	–	–	–	–	–
2 – 5	16	11	3	2	–	–	–	–	–
6 – 10	7	3	1	–	–	–	–	2	1
11 – 20	6	1	2	1	–	–	–	2	–
21 – 30	4	–	–	1	–	1	–	1	1
31 – 40	5	1	2	–	–	–	–	–	2
41 – 50	8	7	1	–	–	–	–	–	–
51 – 60	15	12	1	1	–	–	–	–	1
61 – 70	20	17	1	1	1	–	–	–	–
71 – 83	10	8	2	–	–	–	–	–	–
Total	151	117	16	6	1	1	–	5	5

The figures in the columns show the number of patients.
The sera were examined with boiled antigen.

RESULTS

The pneumonia sera are shown in Table 1. Among the 151 hospitalized patients with pneumonia 34 had a positive *M. pneumoniae* CFT in one or more samples of blood. Twenty nine of these patients had only 1 blood sample or several samples that all were positive with the same titre, whereas 5 patients had a significant rise in titre during the disease.

Sample No. 1	*Sample No. 2*
neg	1:160
neg	1:320
1:10	1:320
1:40	1:160 (sample No. 3 1:320)
1:40	1:640

Five patients had a titre of 1:320 in one sample or in both of two samples and these showed a marked difference from the distribution pattern of the weaker positive reactions. A characteristic finding was the marked predominance of strongly positive reactions and significantly rising titres in the older children and young adults.

The previously published cases of pneumonia (9) are presented in Table 2. There were 23 patients of whom 1 had anticomplementary serum.

TABLE 2

Results of M. pneumoniae CFT in Sera from 22 Hospitalized Patients with Pneumonia in the Oslo Area in the Period 1959–1964

Age in years	Number of patients	*Mycoplasma pneumoniae* complement fixation test						
		Titre in 1 sample or in several samples with equal titres						
		Neg	1:10	1:80	1:160	1:320	1:2560	Significant rise in titre
15 – 20	7	1	2	1	1	2	–	–
21 – 30	6	4	–	–	1	–	–	1
31 – 40	4	2	–	–	–	–	1	1
41 – 50	2	1	1	–	–	–	–	–
51 – 59	3	2	–	–	–	–	–	1
Total	22	10	3	1	2	2	1	3

The figures in the columns show the number of patients.
The sera were examined with antigen extracted with ether.

TABLE 3

Incidence of Positive M. pneumoniae CFT (titre ≧ 1:10) in 632 Blood Donors in Different Age Groups

Age in years	Number examined	Pos. *M. pneumoniae* CFT Number	%
18 – 25	171	22	12.9
26 – 35	175	30	17.1
36 – 45	175	33	18.9
46 – 55	111	27	24.3
Total	632	112	17.7

Out of the 632 blood donors a total of 112 had positive *M. pneumoniae* CFT (titre ≧ 1:10), i.e. 17.7 per cent; 83 had a titre of 1:10, 26 had 1:20 and 3 had 1:40 which was the highest titre found in this group. The age distribution is shown in Table 3. The blood donors were aged 18–55 years and were of both sexes. The incidence of positive *M. pneumoniae* CFT increased with the age of the blood donors: in the oldest group (46–55 years) the incidence was 24.3 per cent, which was nearly twice as high as that in the youngest group (18–25 years) where it was 12.9 per cent. The difference is statistically significant

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0 – 2	60	57	3	–	–	–	–	–	–
2 – 5	16	11	3	2	–	–	–	–	–
6 – 10	7	3	1	–	–	–	–	2	1
11 – 20	6	1	2	1	–	–	–	2	–
21 – 30	4	–	–	1	–	1	–	1	1
31 – 40	5	1	2	–	–	–	–	–	2
41 – 50	8	7	1	–	–	–	–	–	–
51 – 60	15	12	1	1	–	–	–	–	1
61 – 70	20	17	1	1	1	–	–		
71 – 80	10	8	2	–	–	–	–	–	–
Total	151	117	16	6	1	1	–	5	5

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21–30	6	4	–	–	1	–	–	1
31–40	4	2	–	–	–	–	1	1
41–50	2	1	1	–	–	–	–	–
51–59	3	2	–	–	–	–	–	1
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(P < 0 05) Out of the 112 positive blood donors 86 had a positive reaction in sample no 2 as well, i e 77 5 per cent persistency after 3 months Table 4 shows a comparison of the results in the two samples

TABLE 4

112 Blood Donors with positive M pneumoniae CFT (titre ≧ 1 10) in Sample No 1 —Comparison with Results in Sample No 2 Taken after 3 Months

Sample No 1	Sample No 2 Neg	1 10	1 20	1 40	AC	Total
1 10	20	36	26	–	1	83
1 20	5	7	12	2	–	26
1 40	–	–	1	2	–	3
Total	25	43	39	4	1	112

DISCUSSION

In the present study the cases of pneumonia were selected among hospitalized patients of whom the clinicians had considered serological virus examination and/or the cold agglutination test to be indicated in all cases Important criteria determining the selection have included a clinically atypical course of disease unsatisfactory response to therapy lack of demonstrable pathogenic bacteria in the respiratory tract Social factors have also influenced the actual hospitalization

Five patients had a significant rise in titre A further 5 patients had a titre of 1 320 in one sample or in both of two samples and in these patients infection by *M pneumoniae* was also considered to be highly *probable as their titres differed sharply from the general distribution* pattern of the weaker positive reactions (Table 1) and the highest titre demonstrated in the blood donors was 1 40 Out of a total of 161 hospitalized cases of pneumonia 10 were thus considered to be *M pneumoniae* infections The incidence was obviously affected by age as it was much *higher in older children and young adults than in other* age groups *As many as 9 out of the 10 cases were in the age group* 6–40 years which only included 22 patients altogether *M pneumoniae* infection was not demonstrated in any of the 60 children under 2 years old and even the lowest titre 1 10 was only found in 3 of the children In the age group above 40 years only one case of *M pneumoniae* infection was found among 53 patients with pneumonia —The incidence of *M pneumoniae* infections demonstrated in the group of pneumonia patients may be somewhat *lower than the true incidence* as some of the single samples obtained in the course of the disease were not taken at a time that was *optimal for serological diagnosis*

M pneumoniae infections have been investigated using *different sero*logical techniques corresponding to the various stages in the study of the aetiology of *primary atypical pneumonia Eaton et al* (6 7) used

the neutralization test in cotton rats and hamsters with antigen prepared by cultivation of the organism in chick embryos in paired sera from 213 patients with different types of respiratory tract disease They found a significant rise in titre in 52 among 84 patients with primary atypical pneumonia and in 15 among 77 patients with undifferentiated infections of the upper respiratory tract

The indirect fluorescent antibody technique with sections from the respiratory passages of chick embryos infected with Eaton agent as antigen has been used by several authors *Liu et al* (18) investigated 68 patients in whom a diagnosis of primary atypical or abacterial pneumonia had been established and found a significant rise in titre in 54 *i e* in 79 per cent —*Cook et al* (5) investigated 26 patients suffering from primary atypical pneumonia and presenting a significant rise in titre in the cold agglutination test and/or the Streptococcus MG agglutination test and found a significant rise in titre against Eaton agent in 22 cases Out of 69 patients with pneumonia in whom cold and Streptococcus MG agglutination tests were negative and in whom also serological reactions to Q fever psittacosis adenovirus and influenza A and B infections were negative a rise in titre against Eaton agent was seen in 18 patients —In a group of students *Evans & Brobst* (10) found a rise in titre against Eaton agent in 24 2 per cent among 119 cases of lower respiratory tract infection —*Chanock et al* (4) investigated recruits and found a rise in titre against Eaton agent in 161 among 238 patients with pneumonia *i e* in 68 per cent in 28 per cent of cases of other types of febrile respiratory tract disease in 8 per cent in cases of afebrile respiratory tract disease and in 6 per cent in recruits in whom disease of the respiratory tract was absent The same group was used by *Mufson et al* (19) who investigated the clinical features in Eaton agent pneumonia and by *Kingston et al* (17) who investigated the results obtained by treatment with demethylchlortetracycline in cases of Eaton agent pneumonia

CFT with *M pneumoniae* antigen has been used in recent years in several investigations of cases of respiratory tract infection *Jansson et al* (15) examined 246 patients with pneumonia and found a significant change in titre or a titre ≧ 1 64 in a total of 16 per cent the incidence being highest in older children and young adults —*Marmion & Davies* found 10 per cent CFT positive among 112 cases of infection of the lower respiratory tract (published briefly in *Goodburn et al* (12)) —*Biberfeld et al* (1) found evidence of *M pneumoniae* infection in 35 out of 107 cases of pneumonia and in 2 patients with bronchitis among 132 cases of milder respiratory tract infection Out of the total of 37 cases 33 had a significant rise in titre and 4 had a CF titre ≧ 1 256 without a rise *M pneumoniae* infection was found most frequently in older children and young adults —*Sterner et al* (21) examined 73 patients with pneumonia (in 39 of these the diagnosis given was primary atypical pneumonia) and found a significant rise

in titre in 27 an additional 14 patients had a titre ≧ 1 256 without a rise —*Van der Veen & van Nunen* (24) examined 348 recruits suffering from acute respiratory tract infection in whom serological tests to influenza and adenovirus infections were negative in all cases They used both the indirect fluorescent antibody technique which gave a significant rise in titre in 31 recruits and CFT which gave a rise in 26 —*Griffin & Crawford* (14) found a CF rising titre in 25 out of 230 recruits with pneumonia —*Forsyth et al* (11) found a CF rising titre in 7 among 32 soldiers with pneumonia —During an outbreak of acute respiratory tract infections, *Sterner et al* (20) examined 22 children aged 1–12 years in a nursery school and found as many as 9 children in whom a significant rise in CF titre to *M pneumoniae* was seen 4 had high unchanged titres

A large number of investigations of cases selected on the basis of different criteria are thus in agreement in that *M pneumoniae* is an important cause of pneumonia The present study is in support of this conclusion Several of the previous investigations also indicate that *M pneumoniae* plays an aetiological part in non pneumonic respiratory tract infections (3 4 10 13 20) *M pneumoniae* infections do not occur in sharp epidemics but sporadically throughout the year (1 3 5) though fluctuations in prevalence have been demonstrated (10 15 24) *M pneumoniae* seems to spread slowly in the population (4) Family outbreaks have been described (15 16 20)

The characteristic age pattern of pneumonias caused by *M pneumoniae* shown in the present study has previously been demonstrated by *Jansson et al* (15) and *Biberfeld et al* (1) using CFT and by *Grayston et al* (13) who isolated *M pneumoniae* most frequently from older children and young adults with pneumonia *M pneumoniae* thus seems to play an especially important role as a pneumonia agent in older children and young adults

According to previous publications opinions on the significance of *M pneumoniae* infections in small children seem to vary *Chanock et al* (3) using the indirect fluorescent antibody technique found a rise in titre to Eaton agent in 18 of 110 children (mean age 27 months) with lower respiratory tract disease of the types bronchopneumonia bronchitis bronchiolitis or croup The cases were selected on the basis of previous negative results of attempts at virus isolation and of serological tests for influenza parainfluenza and adenovirus infections —*Sussman et al* (22) used CFT for the examination of 106 small children (mostly younger than 4 years) with respiratory tract infections chiefly affecting the lower respiratory passages (76 of the children had pneumonia and/or bronchiolitis) They found a low incidence of *M pneumoniae* CF antibodies as only 1 child had a rise in titre (from < 1 8 to 1 16) 9 children had low stationary titres (1 8 or 1 16) and only 3 children had titres > 1 32 —The present findings in small children are very similar to those obtained by *Sussman et al* (22) in 76 children

under 5 years old and suffering from pneumonia no case of *M pneumoniae* infection could be demonstrated A positive reaction with titre 1 10 or 1 20 was found in 8 (10 5 per cent) this incidence is even lower than that in healthy adult blood donors (Tables 1 and 3) The present findings thus seem to indicate that *M pneumoniae* is of minor importance in the aetiology of pneumonia in very small children

In several of the earlier investigations referred to the results of the cold agglutination test were compared with the *M pneumoniae* CFT (or the indirect fluorescent antibody technique) It was not possible to investigate this aspect in the present study as only some of the samples were sent in for the cold agglutination test and in many of the other samples the serum was separated from the coagulum at refrigerator temperature Several of the authors referred to also present the results of isolation of *M pneumoniae* from patients

In the blood donors a high incidence (17 7 per cent) of weakly positive *M pneumoniae* CFT was demonstrated (Tables 3 and 4) 77 5 per cent of the blood donors who had a positive reaction in the first sample had also a positive reaction in a second sample taken after 3 months This is considered a high degree of agreement especially when the very low titres are taken into consideration It is therefore concluded that in the general population in the Oslo area in 1961 there was a true high incidence of low titre CF antibodies against *M pneumoniae* —*Jansson et al* (15) examined samples taken in 1963 from 484 Finnish blood donors and demonstrated a positive *M pneumoniae* CFT (titre ≧1 8) in 26 (5 4 per cent) These authors found also a pronounced preponderance of weak reactions in that as many as 24 had a positive reaction only in a serum dilution of 1 8 —*Cook et al* (5) examined sera taken in 1957 from 152 individuals aged 0–69 years in a group of the general population in Maryland The sera were tested using the indirect fluorescent antibody technique in a dilution of 1 10 and a positive reaction was found in 25 (16 per cent)

The high incidence of *M pneumoniae* antibodies in the blood donors indicates that *M pneumoniae* is widespread in the population The high incidence also agrees well with the finding by *Chanock et al* (4) that only a very small number of the serologically demonstrable Eaton agent infections resulted in pneumonia

The CFT diagnosis of *M pneumoniae* infections is most reliable when there is a significant rise in titre in paired sera However considering the low titres found in blood donors considerable diagnostic significance can probably also be attributed to a titre of ≧ 1 160 in a single blood sample

SUMMARY

151 hospitalized patients with pneumonia in the Oslo area were examined using *M pneumoniae* CFT In 10 cases evidence of *M pneumoniae* infection was found 5 had a significant rise in titre and 5 had

a titre of 1 320 in 1 specimen or unchanged in 2 specimens 9 out of the 10 cases were in the age groups 6–40 years which included a total of 22 patients No case of *M pneumoniae* infection could be demonstrated in 60 children under 2 years old with pneumonia and the incidence of antibodies in these small children was even lower than that in healthy adult blood donors It is concluded that *M pneumoniae* is an important cause of pneumonia in Norway as well as in other countries especially in older children and young adults but the organism seems to be of minor importance in the aetiology of pneumonia in very small children

A positive *M pneumoniae* CFT with a low titre was found in 112 (17 7 per cent) among 632 healthy blood donors the highest titre demonstrated being 1 40 In 77 5 per cent of the positive blood donors the reaction was also positive in a new blood sample taken after 3 months The incidence of positive *M pneumoniae* CFT increased with the age of the blood donors In the oldest group (46–55 years) the incidence was 24 3 per cent and in the youngest group (18–25 years) it was 12 9 per cent The high incidence of *M pneumoniae* CF antibodies in blood donors indicates that *M pneumoniae* is widespread in the general population

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None of the microorganisms studied had any demonstrable growth at 37 in the cell culture medium supplied with antibiotics No cytopathogenic effect from the microorganisms was observed in the cell cultures

RESULTS

Screening for Microorganisms Exerting VIC

From a sea water sample with a VIC of 4 0 log units 90 microorganisms were isolated A number of these were lost in attempts to sub culture the strains others were discarded being considered as irrelevant due to their terrestrial or fresh water origin The remaining 32 bacterial or fungal strains were divided into 5 pools which were suspended in heat treated sea water so that each pool contained roughly the same number of organisms as judged from optical density measurements

Table 1 demonstrates that two of the pools exerted a VIC of more than two log units The antiviral activity of pool D was lost in attempts to maintain the strains on bacerial substrates at 25 C and in the following experiment interest was focused on the strains of pool C

TABLE 1

The VIC (Virus Ina tivating Capacity) Obtained with 5 Different pools of Marine Microorganisms Suspended in Heat Treated and Filtered Sea Water

Specimen tested	VIC
Pool A	1 5
Pool B	0 8
Pool C	3 6
Pool D	2 6
Pool E	0 5
Untreated sea water	4 0
Heat treated and filtered sea water	0 0

TABLE 2

The VIC (Virus inactivating Capacity) of 4 Marine Ba teria (C 1–C 4) and one Fungus (C 5) Suspended in Heat Treated and Filtered Sea Water

Specimen tested	VIC
C 1	3 5
C 2	0 5
C 3	1 2
C 4	1 5
C 5	0 3
Untreated se ater	3 7
Heat treated nd filtered se w ter	0 1

Pool C consisted of 4 dit rent bacter i ndicated by the morphological appearance and cultural charac u s and one fungus I each strain of microorganism in pool C wa i n sted separately The sus

pensions of the bacterial strains contained about 100 000 cells per ml and that of the fungus about 8 000 spores Table 2 illustrates that the suspension of the C 1 strain alone gave a significant VIC The C 1 bacterium was later identified as *Vibrio marinus* (1)

Influence of Growth Temperature on the Antiviral Activity of the C 1 Bacterium

C 1 bacteria serially cultured at 23 C their optimal growth temperature (1) gradually lost their antiviral character (Table 3) Although the bacterium grew at a lower rate of 4–12 C than at 25 (1) subcultures at the lower temperatures retained their antiviral properties (Table 3) In agreement with these results the twenty fifth subculture at 23 with an insignificant VIC regained its virus inactivating qualities after only one cultivation at 4 (Table 3)

TABLE 3

The relationship between Growth Temperature and VIC (Virus Inactivating Capacity) of the C 1 Bacterium

Subculture No	Growth temperature	VIC
1	23	3 8
2		4 0
5		1 6
9		1 6
25		1 8
50		1 0
1	12	4 [illegible]
2		3 4
3		3 8
4		3 [illegible]
1	4	3 4
[illegible]		3 4
3		3 0
4		3 [illegible]
5		3 0
The 25th subculture at 23 passed once at	4	3 8

The No 1 subculture in the three different series originated from the same c l ns Cultures were grown on fish peptone agar plates (4)

Influence of the C 1 Bacterium on the System for Assay of VIC

If in the presence of the C 1 bacteria the sensitivity of the cell cultures used for assay of virus activity was diminished results mimicking a virus inactivation would be obtained To exclude this possibility virus was titrated on cell cultures to which 100 000 bacteria had been added 24 hours earlier The titres recorded did not differ from those of the controls without bacteria

In another experiment (Fig 1) the incubation times necessary for the appearance of cytopathic changes of cell cultures inoculated with

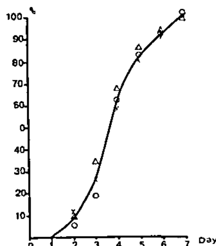


Fig 1

Per cent of virus inoculated cultures showing cytopathic changes plotted against incubation time in days All cultures were inoculated with the same dose of polio virus type 3 suspended either in 0 1 ml of sea water (X) or in suspension of 100 000 C 1 bacterial cells (0) or in Hanks solution (Δ)

the same doses of infective virus are compared Results of more than 500 cultures were analysed One group of cultures had received together with virus also 0 1 ml of sea water a second group received together with virus 0 1 ml of a suspension containing 100 000 bacteria per ml and finally a third group received virus and 0 1 ml of Hanks solution As illustrated by the fig any differences concerning the time required for the appearance of cytopathic changes in the different were not observed Thus the addition of sea water or bacteria did not seem to affect the sensitivity of the cell cultures to viral cytopathogenicity

Comparison between the Kinetics of Virus Inactivation in Sea Water and in Suspension of Bacterium C 1

As reported previously (3) the course of inactivation of poliovirus in sea water is characterized by an initial lag phase and a gradually reduced rate of inactivation during the latter part of the observation period

In the following experiments the C 1 bacteria were cultured on fish peptone agar plates (1) at 4 C Bacterial cells from cultures not older than one week were suspended with poliovirus in heat treated sea water and incubated at 23 C For comparison virus suspended in untreated sea water was studied in parallel Samples were drawn daily for 8 days and titrated for residual virus infectivity Fig 2 indicates that the inactivation of virus in the presence of C 1 bacteria followed the same course as that of the virus suspended in sea water

only at a low growth temperature the formation of the antiviral factors was favoured

SUMMARY

From a sample of sea water 32 marine microorganisms were isolated One of these a bacterium which in the major characters shows resemblance to a marine bacterium referred to as *Vibrio marinus*, was found to possess certain antiviral properties If suspended in sea water depleted of virus inactivating capacity it restored the capacity of the water to inactivate virus This property of the bacterium was maintained only if the bacterium was subcultured at a low temperature (4–12) whereas after a number of subcultures at 25 the antiviral property disappeared The bacteria suspension and the sea water were both sensitive to certain physical and chemical treatments The possible relation of the bacterium to the virus inactivating capacity of sea water is discussed

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4 *Magnusson Sigyn* Unpublished data

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CHARACTERIZATION OF A MARINE BACTERIUM ASSOCIATED WITH VIRUS INACTIVATING CAPACITY

By

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and E. Lycke

Received 11 iv 67

Viruses suspended in sea water will lose their infectivity for cell cultures (7 8) This effect which as yet is referred to as the virus inactivating capacity (VIC) of sea water seems due to factors of organic nature present in the sea water

The VIC of sea water can be eliminated by heating or filtration (7 8) of the water In a previous report (9) it was demonserated that addition of a marine bacterium to heated and filtered water restored the VIC of the water The present study describes some characteristics of the bacterium and discusses its taxonomic position

MATERIALS AND METHODS

Isolation of the marine bacterium A number of 10 ml aliquots of freshly collected sea water were each passed through a Millipore membrane filter type HA (porosity 0 45 µ) The membranes were placed on petri dishes with various types of solid media and were incubated for 5 days at 15 Altogether 32 microorganisms were investigated Out of these 12 different organisms were obtained on a rose bengal agar plate One of the 12 strains referred to as the C 1 bacterium was found to exert a virus inactivating activity *i e* when the bacterium was added at a concentration of about 100 000 cells per ml to sea water from which the VIC had been eliminated by either heating or filtration the VIC of the water was restored

The sample from which C 1 was isolated was taken at th[illegible]th of one meter using a sterile bacteriological water sampler [illegible] The Vală sampler The sampling was made at B[illegible]ttö 10 km WSW of the mouth of th[illegible] The water temperature was [illegible] and the salinity about 1 7 [illegible]

Media The following media have b[illegible] and b[illegible]th media rose bengal agar (14) blood agar [illegible] tion tests citrate broth and medium for V[illegible] and [illegible] (13) The four latter media were prepared with [illegible]ed sea [illegible] carbohydrate media media for indol and urease test [illegible] prepared in 2 per cent NaCl solutions If n[illegible] stated [illegible] were made at 22–25

This study was supported by [illegible] the Sw[illegible] against Polio The skillful technical assistance of M[illegible] is gratefully acknowledged

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only at a low growth temperature the formation of the antiviral factors was favoured

SUMMARY

From a sample of sea water 32 marine microorganisms were isolated One of these a bacterium which in the major characters shows resemblance to a marine bacterium referred to as *Vibrio marinus* was found to possess certain antiviral properties If suspended in sea water depleted of virus inactivating capacity it restored the capacity of the water to inactivate virus This property of the bacterium was maintained only if the bacterium was subcultured at a low temperature (4–12) whereas after a number of subcultures at 25 the antiviral property disappeared The bacteria suspension and the sea water were both sensitive to certain physical and chemical treatments The possible relation of the bacterium to the virus inactivating capacity of sea water is discussed

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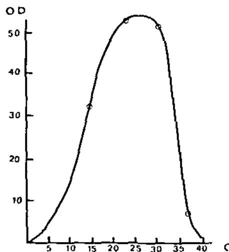


Fig 2

Growth of the C 1 bacterium at different temperatures in fish peptone broth Incubation 24 hrs Optical density (O D) plotted against the incubation temperature

colonies of butyrous consistency They had a strong putrefactive odour In sidelight the colonies appeared iridescent but they did not show fluorescence in UV light and they were not photogenic

Optimal growth was obtained on fish peptone or blood agar plates On blood agar a moderate α haemolysis was observed Fish peptone broth became strongly turbid in 24 hours A flaky sediment was formed when the rather loose surface membrane became ruptured After 5 to 7 days the sediment became black

The organism grew better on media prepared with sea water than on media prepared with distilled water supplemented with 2 per cent NaCl but growth became after a few days good even on the latter media No absolute reuirement for sea water could thus be found

The temperature dependence is illustrated by Fig 2 which shows that optimal growth was obtained at a temperature ranging from 20 – 30° At 4 three to four days were required to obtain measurable growth

Metabolic Characteristics

No difference in growth wa ob erved when incubat on was performed over a p 0 range of 76 to 760 mm Hg (10–100 p r cent 0) Growth was retarded at a p 0 of 16 m m Hg (2 per cent 0) At a p O_2 of 1 6 mm Hg sparse growth howe r could still be observed During aerobic growth nitrate was red c d to nitrite

As seen in Table 1 the bact ium was able to utilize a series of carbohydrates and sugar alcohols for growth with production of acids but no gas

TABLE 1

Carbohydrate Utilization

Carbohydrate	Acid
Glucose	+
Galactose	+
Fructose	(+)
Sucrose	+
Lactose	+
Maltose	+
Threhalose	—
Xylose	+
Arabinose	+
Rhamnose	+
Mannitol	+
Sorbitol	(+)
Dulcitol	—
Adonitol	—
Inositol	—
Inulin	(+)
Dextrin	(+)
Salicin	(+)
Esculin	—

\+ positive reaction after 48 hrs
— negative reaction after 48 hrs
(+) dubious reaction after 48 hrs

TABLE 2

Sensitivity to Antimicrobial Drugs at 23

Drugs	MIC (mcg or IU per ml)
Sulpha	1 100 §
Bensylpenicillin	8 0 §
Ampicillin	1 5 §
Tetracycline	≤0 01
Chloramfenicol	0 1
Streptomycin	1 0
Erythromycin	0 2
Neomycin	≤2 0
Gentamycin	10 0
Nitrofurantoin	≤10 0
Nalidixinic acid	≤6 3
Cephalosporin	≤2 0
Kanamycin	>30 0

The tests were performed according to the standard method of *Ericsson et al* (3)
§ Sparse growth was observed also within the zone corresponding to the MIC value listed in the table
MIC minimal inhibitory concentration

Starch was hydrolysed but this was evident only after more than 72 hours of incubation Agar and cellulose were not digested Growth on gelatine was moderate but with rapid crateriform liquefaction Citrate could be used as a carbon source Selenite medium gave scanty growth

Catalase test (11) was positive although evolution of oxygen from

H O was not vigorous The oxidase and urease tests were positive Voges Proskauer and methyl red tests were negative Indole was not produced Hydrogen sulphide production could not be demonstrated with lead acetate paper (11)

Sensitivity to Antibiotics

As seen in Table 2 the bacterium was sensitive to most antibiotics tested However with sulphonamide bensylpenicillin and ampicillin sparse growth was observed also within the zone corresponding to the MIC-value listed for these antibiotics in the table No sensitivity was found to kanamycin

DISCUSSION

The morphological characteristics of the bacterium studied agree well with the description given for the family Pseudomonadaceae in *Bergey s* Manual (1) The predominance of curved cells in the organism seems to justify its placement among the vibrios a genus which includes several marine species In spite of the ability of the organism to proliferate at relatively low oxygen pressures it should be classified as fundamentally aerobic

Positive characters which are considered to be of taxonomic importance for the present bacterium were gelatin liquefaction production of nitrite from nitrate a positive urease test starch hydrolysis and chromogenesis negative characters of consideration would be the lack of hydrogen sulphide formation the negative indol Voges Proskauer and methyl red tests and the lack of action on agar and cellulose Less useful is the carbohydrate fermentation pattern since most common corbohydrates are fermented with acid production but no gas The antibiotic sensitivity pattern considered to be of value in taxonomic studies of marine bacteria (12) unfortunately is of little help too since older descriptions of vibrios do not contain data on antibiotic sensitivity

The most comprehensive studies of marine vibrios have been made by *ZoBell & Upham* (14) *Humm* (5) and *Kadota* (6) but the bacterium described in the present report does not seem to be identical with any of the species described b[illegible] these authors The [illegible]ib[illegible]os listed by Kadota are outstanding in being cellulose [illegible] [illegible]rs and those of Humm (with one exception) liq[illegible]fy o[illegible] other[illegible] [illegible] agar However the bacterium seems to be rel[illegible] to an org[illegible] *[illegible] Marinus* isolated by *Russell* (13) in 189[illegible] f[illegible]m the Gulf of [illegible] and originally given the name *Spirillum mar[illegible]* The taxono[illegible] position of this bacterium was later emende[illegible] by *[illegible]rd* (4) who placed it with the vibrios The organism has recently been reisolated and studied by *Colwell & Morita* (2) Among other characteristics its antibiotic sensitivity pattern has been described In the major characters the bacterium discussed here resembles *Vibrio marinus*

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PRODUCTION OF ANTIBIOTICS BY EPIDERMOPHYTON FLOCCOSUM

I The Antibiotic Spectrum of Crude Filtrate

By

ALF WALLERSTRÖM

Received 19 iv 67

Though it was known already before the antibiotic era that *Trichophyton* fungi possess antibacterial activity (*Nakamura* 1932 *Honda* 1936) only few detailed studies of the formation of antibiotics by dermatophytes have been published Some of the sparse publications report the behaviour of strains of *Epidermophyton floccosum*, it is striking that this fungus always was found to be active in this respect despite the variety of testing methods used *Patiala* (1947) thus reported it to inhibit staphylococci cultivated together with it in liquid medium *Vonkennel et al* (1943) appear to have been the first to have extracted inhibiting substances (Mycoine) from cultures of dermatophytes *inter alia Epidermophyton* fungi The production and extraction of such substances was further investigated by *Robbins et al* (1945) *Ciferri* (1947) *Yamasaki* (1947) and *Urabe* (1951) *Peck & Hewitt* (1945) believed the antibiotic factor of the dermatophytes to be a penicillin like substance mainly because of its spectrum of activity and behaviour toward penicillin resistant organisms *Uri et al* who later (1955) demonstrated the production of benzyl penicillin by *Trichophyton mentagrophytes* found that strains of *Epidermophyton floccosum* inhibited not only staphylococci but also *E coli* (16)

Nishio (9) studied *Epidermophyton floccosum* in shake cultures using modified Czapek Dox medium and found antibiotic activity against *inter alia staphylococci various members of* [illegible] *Bacillus and Corynebacterium diphteriae* By extraction with org[illegible] ents and column chromatography he obtained from [illegible] celium [illegible] s from culture broth thr[illegible] fractions including two describ[illegible] penicillin and a penicillin [illegible]ke substance the third fraction w[illegible] [illegible]ned to the actinomycin group *Katagiri & Sato* (6) found this th[illegible]d traction to inhibit staphylo[illegible] ci *Sarcina Corynebacterium* and though not so strongly *Bacillus* it had a weak inhibitory effect on pneumococci haemolytic streptococci and tubercle bac[illegible] and no effect at all on *Enterobac*

teriaceae Pseudomonas aeruginosa yeasts and moulds. It had no demonstrable effect on *Ehrlich's* ascites carcinoma cells or HeLa cells.

The present investigation was prompted by the observation of inhibitory zones around dermatophyte colonies in cultures with simultaneous growth of staphylococci. In a pilot study strains of dermatophytes were cultivated submerged in shake culture. *Trichophyton mentagrophytes*, *Trich. rubrum*, *Trich. verrucosum*, *Trich. terrestre*, *Microsporum canis* and *Keratinomyces ajelloi* were found to be inactive or at most slightly active, whereas *Epidermophyton floccosum* regularly produced antibiotic substances which accumulated in the culture medium. The antibiotic spectrum of this fungus was studied as it appears when tested with untreated crude filtrate.

MATERIAL

Fungi. 50 strains of *Epidermophyton floccosum*, including 2 type strains received from other laboratories, and strains isolated from material sent to the Bacteriological Department, Lund Hospital, were tested for antibiotic activity.

Bacteria. All the fungi were tested against a variety of bacteria, mainly strains recently isolated from routine laboratory cultures and selected at random. Only in a few cases (*Corynebacterium diphtheriae*, *Clostridium*, *Lactobacillus*, *Listeria*) were older or freeze dried strains used.—Oxidase positive gram negative cocci producing acid from glucose, maltose and laevulose were grouped together under the species name *Neisseria pharyngis* (*Cowan & Steel* 1965), those not producing acid from any of these sorts of sugar within 2 days *Neisseria catarrhalis*.

Substrates

For isolation and maintenance purposes the fungi were cultivated on Sabouraud's glucose agar (Oxoid). For production of antibiotic substances all strains were cultured in liquid medium of the following composition:

casein hydrolysate Difco	1
glucose	1%
NH_4NO_3	1‰
KH_2PO_4	5‰
$MgSO_4 \cdot 7H_2O$	1‰

pH 5. Trace metals were added as a partial EDTA chelate (for details [illegible] *Falmstierna & Rije* (10)).

Most strains were cultured on this medium within 6 weeks after their first isolation (5 strains had been maintained in the lab. cult. eg. for 7, 12 months [illegible] (or more than one year). As soon as possible after the fungi had grown [illegible] serving cultures were prepared, i.e. comminuted mycelium was mixed with sterile oil and sterile sand respectively.

The term "filtrate", unless otherwise stated, applies here to sterile filtrate from cultures grown on the above mentioned casein hydrolysate medium.

For special purposes media of the following composition were used:
modified Czapek Dox medium

glucose	4
$NaNO_3$	0.3
NaH_2PO_4	0.1%
$MgCl$	0.05%
$NaCl$	0.05%
proteose peptone Difco	1

malt extract (Difco) 179
mycological peptone (Difco) 3

pH 5 2

In most cases 0 1 per cent cycloheximide (actidione) was added to the cultures in order to prevent contamination with moulds gr wth of which was found markedly to lower the yield of antibiotic substances The presence f cycloheximide itself was found not to influence the yield.

Medium for testing of antibiotic activity most of the tests were carried out on Oxoid DST agar Streptococci pneum cocci *Neisseria pharyngis* and *Neiss catarrhalis* were studied on plates containing Oxoid Blood Agar Base No 2 and 4 per cent horse blood *Haemophilus* and *Neiss meningitidis* were studied on 7 per cent haematin agar (Oxoid) and *Neiss gonorrheae* on a special medium in CO atmosphere (13)

METHOD

For production of antibioti substance all the *Epidermophyton* strains were grown submerged in shake cultures at room temperature (19) The flasks were inoculated with comminuted mycelium from 1–3 week old Sabouraud glucose agar cultures The inoculum u ed wa about 0 5 cm of the mycelium carpet per 100 ml medium Samples of the culture broth were taken with a sterile pipette at intervals of 1–3 days and tested for antibiotic acti it against one and the same reference strain of *Staphylococcus albus* The fermentati n was interrupted when the concentration of antibiotic substances did not seem to increase further i e 8–12 days The broth was then filtered through a Seitz Fl (bef re further investigation) Filtrates tolerated storage at —20 C with only slight lo of activity and except for those containing a penicillin like comp nent also at r m temperature or in the refrigerator pH in the culture broth invariably rose during the growth of the fungi from originally 5 to v lues between 7 7 and 8 2

The antibacterial activity was tested by the plate diffusion technique cylindrical holes 8 mm in diameter were punched out in plates previously sown or streaked with bacteria (see b low) and the holes were then filled with culture filtrate from the fungus The nhibition zones were mea ured after 1? ?0 hour incubation of the plates at 37 C. Two diffe ent method of ino ulating the plates were used

Metho l 1 the plate was inoculated with radial streaks from undiluted br th cultures and a hole to be filled with filtrate was punched out in the centre (the radial streak meth d adapted from Mohs 1945)

Metho l 2 the surface of the medium as flooded with a susp nsion of bacteria in normal saline or broth the excess fluid removed and the plates briefly dried The concentration of bacteria in the suspension was chosen so as to give a den e but not confluent growth of bacterial colonies (*Eriksson* 1951 *Ericss n* 1960)

Method 2 w s used for *Cl stri lium* and *B illus* p and for *Neiss g norrho ae* As for other *Neisseriae C rynebacterium β* streptoco ci and p um cocci the investigation was started with metho l 2 but as this method as not sensitive enough to register low grade activit in the filtrate (which was found desirable in the study of the e bacteria) method 2 was l ter preferr d. Oth r ba teria and ea ts were tested with method 1 for comparis n me sta h l l tr n were t sted with method 2 as well It was found that f lt tes g n tion zones l ss than 15 mm in diameter with meth d 2 l n t w nt be exp t d to give inhibition when metho l 1 was used diff n m r rat 3 hours before incul ation at 37 C pr duc ign f t th e of the z nes The standar l deviation as l from 1 l f one and the same filtrate was $\sigma \pm 1$ 3 n m 1 4 mm

The susceptibility of the b ct antib t praised with antibiotic impr gnated p per disc 5 eter are l d from Bakt riologiska lab ratoriet Karolinska Sjukhu l lm The di t ined alpha 2 4 mg benzyl penicillin 20 IE strept ampi ll 20 IE tetracy line 50 mcg chloramphenicol 30 mcg kanat in t vth m 0 mcg novobiocin 50 mcg rist cetin 100 mcg methicill n lin 50 m g

Bact ri l tr ins h ing n h b t n z ne more than 15 mm in di m ter when tested n floo led plate are in th f ll wing arbitr l d ign te l s "s nsitiv " to *Ep lern phyton* filtrate

less sensitive to the first *Epidermophyton* filtrate were also less sensitive to the other tested filtrates while other staphylococci showed inhibition zones of largely the same size

In a third part of the investigation filtrates of a further 46 strains of *Epidermophyton* were tested for their activity against staphylococci and other bacteria All the strains proved to be active against staphylococci 25 of the fungal strains were tested against those bacteria with the remaining 21 strains further testing of the antibiotic spectrum was carried out with a smaller number of representative bacterial strains (β streptococci enterococci pneumococci *Corynebacterium diphteriae* *Listeria monocytogenes* *Neiss pharyngis* and *Neiss catarrhalis* as well as sensitive and insensitive staphylococci) Each fungal strain was tested against a total of 8–24 bacterial strains The activity also of these fungi showed the previously observed pattern but 9 of the fungal strains (like test strains 2 and 3) also had a certain activity against pneumococci particularly when fresh filtrates were tested some of these fungal strains also showed a slight inhibition of β streptococci The activity against these two bacterial species disappeared or was much reduced on addition of penicillinase to the filtrates

In view of these findings the effect of addition of penicillinase to the filtrates of 9 *Epidermophyton* strains was studied As test bacteria a strain of *Staphylococcus albus* and a strain of *Neisseria catarrhalis* both sensitive to penicillin were used

TABLE 4

Effect of Penicillinase on Filtrates of Epidermophyton Floccosum
Test Bacteria Staph Albus Neiss Catarrhalis

Epidermophyton strain No	Zone diameter Staph albus			Zone diameter Neiss cat			Zone diameter pneumococci
	untreated filtrate mm	treated filtrate mm	reduction mm	untreated filtrate mm	treated filtrate mm	reduction mm	mm
16	24.3	19.0	−5.3	19.0	15.0	4.0	3
19	22.0	17.0	−5.0	15.0	12.0	3.0	2
12	21.0	14.0	−7.0		not done		19
20	23.5	19.0	−4.5	18.0	16.0	2.0	18
14	23.5	19.8	−3.7	19.5	13.0	6.5	13
13	19.5	14.0	−5.5	15.0	3.0	12.0	12
15	26.5	24.0	−2.5		not done		1
18	18.5	15.8	−2.7	16.5	17.0	−0.5	0
1	29.0	27.5	−1.5	25.5	[illegible]	−1.5	0
Control	25.5	0	−25.5	33.0	0	−33.0	

For comparison the filtrates are listed in relation to their activity against pneumococci A solution containing 20 IU benzyl penicillin/ml was used as a control

In Table 4 the findings are listed in relation to the activity against pneumococci On the average the greatest loss of activity on addition of penicillinase was found for those strains that showed the highest

activity against pneumococci Filtrate of test strain 1 showed no loss of activity after treatment with penicillinase —Addition of penicillinase also reduced the activity of two filtrates against a strain of *Corynebacterium diphtheria* and a strain of *Clostridium sporogenes*

For comparison a few *Epidermophyton* strains were cultured also on modified Czapek Dox medium the antibiotic spectra were the same but their effect on pneumococci was greater than when the same strains were cultivated on casein hydrolyzate Test strain 1 which when cultivated on casein hydrolyzate or Czapek Dox had no demonstrable effect on pneumococci showed some activity (zone diameter 10 mm) when the fungus was cultivated on malt peptone medium

All of the *Epidermophyton* strains examined showed some degree of activity against staphylococci But 11 strains were poor producers of antibiotics in the medium used the activity proved equally low against staphylococci as against diphteria bacilli and *Neisseria catarrhalis* After having been kept on Sabouraud medium for a longer time (more than six months) the activity of some strains diminished and in one case it disappeared completely In one case a fungus cultured on Sabouraud medium suddenly lost its activity 2 years after the first isolation while the activity was found to persist in a soil culture of the same strain started 6 months previously

The staphylococcal material included strains which when tested routinely proved to be resistant to one or more of the following chemotherapeutics sulpha streptomycin tetracyclins chloramphenicol kanamycin and erythromycin also these strains were however sensitive to *Epidermophyton* filtrate Neither did the size of the inhibition zones of benzyl penicillin ampicillin meticillin novobiocin or ristocetin show any correlation suggesting cross resistance On the other hand with fucidin cross resistance could be established 3 staphylococcal strains insensitive to *Epidermophyton* filtrate were only slightly sensitive to this antibiotic 2 of these were also slightly sensitive to tetracycline The significance of these observations concerning cross resistance will be investigated further

DISCUSSION

Production of antibiotic substances [illegible] such a common property of *Epidermophyton floccosum* that it [illegible] be assumed that all strains of this fungus are potential producers of [illegible] antibiotics As th[illegible] substances formed are active against *inter alia* st[illegible] cci and diph[illegible] oids they should if they are formed by *Epider[illegible]* also in its parasitic state be able to facilitate the establis[illegible] the fungus in the human skin by eliminating parts of the norm[illegible] flora

A striking feature of the fung[illegible] is its activity against staphylococci in a non selected material o[illegible] 3 out of 466 staphylococcal strains (0 6 per cent) showed reduced sensitivity to *Epidermophyton filtrate* [illegible]

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ISOLATION OF *TOXOPLASMA GONDII* FROM THE FLESH OF SHEEP, SWINE AND CATTLE

By

KRESTEN WORK

Received 2 ii 67

More than fifty years have elapsed since *Nicolle & Manceaux* and *Splendore* described the protozoan parasite *Toxoplasma gondii* and extensive studies especially during the past two decades have shown how widespread an infection toxoplasmosis is

Toxoplasma has been demonstrated all over the world in all mammals hitherto examined and in a great number of birds but epidemiologically attention has been paid mostly to animals living in close contact with man and an extensive amount of literature now exists dealing with toxoplasmosis in domestic animals

Sheep

Toxoplasmosis in sheep has been *reported in a number of countries* but [illegible] in Denmark In 1949 *Olafson & Monlux* described toxoplasmosis in a sheep in the State of New York The animal had difficulty in moving about the muscles were rigid and the legs were held in a semi flexed position There was marked dyspnea with [illegible] nasal discharge On post mortem examination there was diffuse non suppurative *encephalomyelitis with slight meningitis and morphologically typical toxoplasma*

A similar case was reported from Australia in 1950 by *Wickham & Carne* Clinically the animal showed signs of circling disease Sections of brain [illegible] and perivascular infiltration and toxoplasma cysts

Attempts to isolate the parasite were not made in these two cases

In 1951 during the lambing season a survey was conducted in New Zeal[illegible] *Hartley et al* (1954) to investigate the economic importance of lamb [illegible] infectious abortion In some of the diseased animals there was a constant [illegible] characteristic lesion in the cotyledons and membranes consisting of numer[illegible] white nodules 1-3 mm in diameter On microscopical examination intracellular tox[illegible] plasma like bodies were seen *No microorganisms could be grown* [illegible] bacteriological media inoculated with material from either membranes [illegible] lambs The interim name of New Zealand type II Abortion was given [illegible] to distinguish it from Type I Abortion caused by a Brucella mutant When in 1957 *Hartley & Marshall* succeeded in isolating toxoplasma from the membran[illegible] name was replaced by toxoplasmosis

Cole et al (1954) reported toxoplasmosis in a flock of 50 ewes and 68 lamb[illegible] ewes *and three* young lambs died with symptoms referable to the respiratory and central nervous systems The diagnosis was established on the basis of micr[illegible] lesions containing toxoplasma [illegible] isolation of toxoplasma by mouse inoculation and toxoplasmin dermal sensitivity

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Aided in part by grants to the *Department of Toxoplasmosis & Viral Disea*[illegible] *King Christian X Foundation* and U S *Public Health Service* Research Grant [illegible] sion (AI 01741) Bethesda Md U S A

In 1959 *Osborne* reported abortion in two flocks of ewes in Australia. One flock consisted of 1000 ewes of all ages and 16 abortions were noted. The other flock was located 10 miles away and consisted of 120 two year old ewes with 14 abortions. Only three of these cases were examined and the lesions present on the foetal cotyledons were identical with those seen in toxoplasma abortion as described in New Zealand. Groups of typical toxoplasma were seen in microscopical sections in one case. No attempts were made to isolate the parasite.

Another case of abortion in Australia was reported in 1959 by *Studdert & Johnson* in a flock of 312 ewes. The foetus was about 120 days old and showed early mummification. The foetal membranes were retained. On removal the cotyledons had multiple white foci 1–3 mm in diameter. Histologically there were numerous areas of necrosis with central calcification. Intracellular toxoplasma was found especially at the periphery of these areas. At about the same time seven other perinatal deaths occurred in the flock in question. According to the authors these could have been due to toxoplasmosis but no investigations were made to confirm that possibility.

In England *Beverley & Watson* (1959) examined foetuses from 39 abortions in 93 flocks by inoculation of brain and liver suspensions in mice. Toxoplasma was found in six cases, five times in the brain and once in the liver. In none of these cases was any other specific pathogen found. In 1960 toxoplasma was isolated from foetus or membranes of 18 out of 192 examined abortions (*Beverley & Watson* 1959, 1961, 1962).

Three outbreaks of toxoplasmosis involving 155 out of 845 Yorkshire sheep were described by *Watson & Beverley* in 1963. The parasite was isolated in 23 out of 33 instances from foetal brain or placenta or both. Serological tests showed evidence of widespread toxoplasma infection in all these flocks, suggesting that infection in pregnancy did not necessarily cause abortion.

Reports of serological studies of sheep are few and not very extensive. *Feldman & Miller* (1956) found 56 per cent of nine Kentucky sheep and five per cent of 66 Navajo sheep positive with dye test titres of 1 : 16 or higher. *Roever-Bonnet* (1957) examined 92 non-inactivated sera from sheep in Amsterdam and found 14 with a titre of 1 : 16 or higher in the dye test. *Carnham & Lainson* (1960) found no positive case among six lambs. *Beverley & Watson* (1961) showed that 89.5 per cent of normal sheep in Yorkshire were positive, 66 per cent having a titre of 1 : 16 or higher in the dye test.

Summary. Toxoplasmosis in sheep seems to run a subclinical course in the majority of cases. Only a few cases with involvement of the respiratory and central nervous systems have been reported.

The incidence varies in different geographical areas with a maximum of 89.5 per cent positives among sheep in Yorkshire, England.

Beyond doubt toxoplasma is an important factor in ovine perinatal mortality and the infection is the cause of heavy lamb losses in New Zealand.

Swine

The first report of toxoplasmosis in swine was published in the U.S.A. in 1952 by *Farrell et al*. Toxoplasma was found in microscopical sections from 8 out of 11 animals from one day to one year old; from two of these the parasite was isolated by mouse inoculation from liver, spleen or heart.

In Denmark *Momberg-Jorgensen* (1956) described an outbreak of toxoplasmosis in a heard of swine. A litter of 11 [illegible] pigs [illegible] ill at the age of one week and six died in the course of a few d[illegible] wing [illegible] ng involvement (especially dyspnoea). The post mortem fin[illegible] s pneumonia, necrotizing lymphadenitis [illegible] nd focal hepatitis, nephritis and splenitis. Th[illegible] f tox[illegible] demonstrated by histological examination in all th[illegible] ch change [illegible] and by inoculation into mice. On serological exami[illegible] f the [illegible] weak positive reaction [illegible] a few sera [illegible] 1 : 50 [illegible]

In 1958 *S[illegible] et al* rep[illegible] [illegible] swine in Japan. Marked changes were found in all the [illegible] the lungs [illegible] inflammatory and o[illegible] matous while the liver showed [illegible] turation a[illegible] infiltration of round cell. Enlargement [illegible] hyperaem[illegible] [illegible] hage and [illegible] tered focal necrosis were found in the spleen. While [illegible] necrosis of the lymph node was characteristic finding. T[illegible] [illegible] demonstrated in the le[illegible] in every case

original method After centrifugation the sediment was suspended in saline to a volume of 10 ml and inoculated intraperitoneally into 10 mice Each mouse was given 1 ml of the inoculum

For every three mice used for inoculation one mouse was used as a control of possible spontaneous infection according to the routine method in this laboratory

The mice were observed for six weeks Any mice that died during this period were examined by means of fresh smears of brain and if present peritoneal exudate At the end of six weeks the mice were bled from the tail and the serum specimens were tested in the dye test for the presence of toxoplasma antibodies Crush smears of brains from all mice were examined microscopically for the presence of toxoplasma cysts

Subinoculations have not been made since it has been shown that these do not result in isolation of toxoplasma from serologically negative laboratory mice (Siim 1961)

Serological Examinations

All blood samples were tested for the presence of toxoplasma antibodies in the dye test The test was performed as indicated by Sabin & Feldman and as standardized by Aagaard for routine use All sera were inactivated for 30 minutes at 56 C All the titres reported are final dilution titres

RESULTS

Table 1 presents the results of the serological examination of 31 sheep 199 swine and 211 cattle The highest rate of infection was found among sheep where 58 0 per cent were serologically positive while 35 2 per cent of swine and 11 4 per cent of cattle were positive with titres of 1 10 or higher Also the individual titres appear to be higher among sheep where nine out of 18 serologically positive animals had a titre of 1 50 or higher while only one of 24 positive cattle had a titre of 1 50

TABLE 1

Serological Examination of Cattle Swine and Sheep

	Number	Negative <1 10	Positive	Dye Test Titre 10	50	250	1250	6250
Cattle	211	187 (88 6)	24 (11 4)	23 (10 9)	1 (0 5)			
Swine	199	129 (64 8)	70 (35 2)	45 (22 6)	20 (10 1)	5 (2 [illegible])		
Sheep	31	13 (42 0)	18 (58 0)	9 (29 0)	4 (12 9)	3 (9 7)	1 (3 2)	1 (3 2)

Figures in brackets per cent

70 swine were serologically positive 45 had titres of 1 10 and 25 had titres of 1 50 or higher Strongly positive sera were found only among sheep where two animals had titres of 1 1250 and 1 6250

Table 2 shows a grouping with regard to age of 180 cattle 31 of 211 were excluded because of lack of information concerning age This table shows that most of the serologically positive individuals are found among the younger animals 23 9 per cent of sera from ¾ 2 year old cattle were positive while only 7.5 per cent were positive in the 2 3 years age group Of 3 to 4 year old cattle 5 0 per cent were positive and over 4 years only 3 8 per cent were found to be serologically positive

TABLE 2

Serological Results in 180 Cattle Grouped According to Age

Age (years)	Number	Dye Test Titres					
		<10	10	50	250	1250	6250
3/4–1	67	51 (76.1)	15 (22.4)	1 (1.5)			
2	53	49 (92.5)	4 (7.5)				
3–4	34	32 (94.1)	2 (5.9)				
>4	26	25 (96.2)	1 (3.8)				

Figures in brackets per cent

A similar grouping was made for swine and the results are shown in Table 3 199 swine are divided into two groups one comprising pigs slaughtered at about six months of age and the other comprising sows and boars which are usually slaughtered when three or four years old In the older group 50 0 per cent are positive as against 23 0 per cent in the younger group The individual titres appear to be higher among younger animals

TABLE 3

Serological Results in 199 Swine Grouped According to Age

Age	Number	Dye Test Titres					
		<10	10	50	250	1250	6250
6 months	109	84 (77.0)	9 (8.3)	1 (11.0)	4 (3.7)		
3–4 years	90	45 (50.0)	36 (40.0)	8 (8.9)	1 (1.1)		

Figures in brackets per cent

A supplementary survey of 55 horses 32 cattle and 39 sheep belonging to Statens Seruminstitut was made and the results are shown in Table 4 Only one horse was found to be serologically positive with a titre of 1 10 in the dye test 25 per cent of cattle were positive all with a titre of 1 10 whereas 64 per cent of sheep were positive

TABLE 4

Serological Examinations of Horses Cattle [illegible] Sheep from Statens Seruminstitut

	Number	Negative <1 10	Positive	Dye Test Titres				
				10	50	250	1250	6250
Horses	55	54 (98.2)	1 (1.8)	1 (1.8				
Cattle	3[illegible]	24 ([illegible]5.0)	[illegible] ([illegible]5.0)	8 ([illegible] 0)				
Sheep	39	14 (36.0)	[illegible] (64.0)	7 (1[illegible].9	13 [illegible]	5 (1[illegible].8)		

Figures in brackets per cent

Isolation of toxoplasma was attempted from a total of 31 sheep 29 swine and 30 cattle The results are shown in Table 5 Isolation was possible from 7 sheep and 10 swine but from none of the cattle It

TABLE 7

Titration of Digested Muscle

Dilution	Number of Mice	
	Inoculated	Positive
Undiluted	10	8
1:10	9	[illegible]
1:100	[illegible]	1
1:1000	9	0
1:10000	10	0

and swine. Cattle, however, appear to a great extent to be negative or to have only low titres.

Distribution of swine and cattle with regard to age was made in order to see whether serologically positive individuals were to be found mainly among younger or older animals. In human populations the percentage of positives is known to increase with age until about 40 or 50 years (*Feldman & Miller* 1956).

This seems to be true also for swine, where most positives are found among the older animals. However, for cattle the results are different, most of the positive sera coming from the group of young animals. No definite explanation of this phenomenon can be given, but it raises the question regarding the specificity of low titres in cattle. *Jacobs* and others claim that sera from cattle should be inactivated at 60° C instead of 56° C to eliminate unspecific positive reactions. Unfortunately *this was not done in the present survey, but the fact that no isolation* was possible from beef might indicate that these titres are not specific. On the other hand, *Jacobs & Remington* isolated toxoplasma from cattle in one case, showing that it is possible for cattle to become infected with toxoplasma. Further studies on inactivation and isolation would probably solve the problem.

The peptic digestion technique seems suitable for isolating parasites from relatively large samples. Trypsin can be used instead of pepsin and has the advantage that trophozoites will survive as well as cyst forms, but it seems to be more toxic to mice than pepsin (*Jacobs* 1960). In this study pepsin was used since the purpose was to demonstrate encysted organisms.

In the present study an isolation trial was only accepted as positive if the mice became positive in the dye test and cysts could be demonstrated in the brains. None of the control mice was positive on serological examination, and no cysts were found on microscopical examination.

In this study isolation was made from one swine with a titre of 1:10. In other studies toxoplasma was even isolated from serologically negative animals. *Jacobs & Melton* (1952) isolated toxoplasma from three pigeons with titres < 1:16 in the dye test. *Jones et al.* (1957)

were able to isolate toxoplasma from a cat with a negative reaction in undiluted serum However in evaluation of these results it must be taken into consideration that the titres reported are original titres and not final dilution titres

The experiments performed to evaluate the number of cysts in meat cannot of course form the basis of any precise statement since the meat suspension was fairly coarse and single meat particles might have contained several cysts However it seems reasonable to conclude that the number of viable cysts was relatively small

Titration of the sediment after peptic digestion shows that a considerable number of parasites survived two hours of peptic digestion This supports the results of *Weinman & Chandler* (1956) that toxoplasma will survive a normal human gastric passage

It can therefore be concluded that the eating—and handling—of raw meat must involve a risk of infection

SUMMARY

A serological and parasitological survey was made of 31 sheep 199 swine and 211 cattle Toxoplasma antibodies were demonstrated in the Sabin Feldman dye test in 58 0 per cent of sheep 35 2 per cent of swine and 11 4 per cent of cattle By means of the peptic digestion technique developed by *Jacobs & Remington* toxoplasma was isolated from 7 sheep and 10 swine It appears that the probability of isolating toxoplasma increases with the titre of the animal in question but even animals with low titres may have toxoplasma in their flesh

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HOST COMPONENTS IN PURIFIED INFLUENZA VIRUS

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Electron microscopic studies have shown that the influenza virus particle matures at or near the surface of the propagating cell (1 17) Investigations indicate that the outer lipoprotein envelope of the virion is derived from constituents of the cytoplasmic membrane (1 11 16 17) Great efforts have therefore been made to establish whether and to what extent host components are acquired by the virus in an unaltered antigenic form

Serological cross reactions between different types of influenza virus have been demonstrated both in the complement fixation (CF) (20) the precipitation (13) and in the haemagglutination inhibition (HI) test (2 12) In the latter test the cross reaction was found to be due to the presence in the virus of a host tissue component which was also found in non infected chicken allantoic fluid (2 3) and in chicken bile (4) This normal allantoic material now designated the host antigen has been purified from chicken allantoic fluid (6) It was found to be a sulphated mucopolysaccharide (7) the monosaccharide composition showing a great resemblance to that of human blood group substances It has been established beyond doubt that the host antigen constitutes an integral part of allantoically grown influenza virus (3 9 15)

Several other host tissue components *viz* Forssman and mononucleosis antigens human blood group A substance and a protein antigen have been demonstrated in influenza virus and other myxovirus preparations purified from chicken allantoic fluid (14 19 21 22) The host antigen can be demonstrated by the HI test The presence of the other components has hitherto been evidenced only by the results of immunization antibody absorption and CF i e activities not directly ascribable to the virus particle proper The crucial point in the evaluation of these results is consequently the purity of the virus preparations with regard to host tissue contaminations

In the present study allantoically cultivated influenza virus has been purified by adsorption to an elution from guinea pig erythrocytes These cells unlike chicken cells contain no Forssman blood group A or mononucleosis antigen The purified virus has been employed in the HI test with various host tissue antisera in absorption experiments and

for immunization of rabbits Different tests for cross reactivity between the host antigen and blood group substances are also included

MATERIALS AND METHODS

Viruses The PR8 strain of *influenza A virus and the Lee strain of influenza B virus* were propagated in the allantoic cavity of 10 day chicken embryos of the *White Leghorn* strain

Purification and concentration of virus Allantoically grown virus with an HA titre of 1 512 was purified and concentrated by adsorption to and elution from guinea pig erythrocytes Two *adsorption elution cycles were performed including* thorough washing of the erythrocytes after adsorption of the virus in the cold Checks for allantoic fluid contaminants were made by the HIB test (see below) for the host antigen The HIB titre of the original allantoic fluid was 1 256 while the last washing gave a negative HIB reaction The final virus eluate showed a haemagglutination titre of 1 6 400

Purification of virus on chicken erythrocytes followed the same lines

Erythrocytes After three washings suspension was made in saline buffered by 0 01 M phosphate at pH 7 2 (PBS) Bovine erythrocytes were treated with trypsin (Crystalline Trypsin Novo) according to the directions given in (23) Chicken erythrocytes were treated with crude RDE (*Cholera filtrate Philips Duphar Amsterdam*) diluted 1 5 or 1 10 for 3 hours at 37 C

Antisera The following antisera against influenza virus were used

Anti PR8 allantois R76 and R77
Anti Lee allantois R57
Anti PR8 chicken erythrocyte eluate R925
Anti Lee guinea pig erythrocyte eluate R85

All antisera except R925 were produced in rabbits by subcutaneous inoculations of the virus in Freund s complete adjuvant Two injections were given at a 14 day interval *The animals were bled after another 14 days Antiserum R92*5 was produced by several intravenous injections

Rabbit antiserum to purified host antigen (R81) and to non infected allantoic fluid (R87) were obtained by subcutaneous injections using Freund s complete adjuvant and following the same schedule as above

The Freund s adjuvant was prepared with tubercle bacilli which had been cultivated on an egg free medium (Sauton)

Two anti Forssman sera against boiled sheep erythrocytes (R925) and against a boiled guinea pig homogenate (R826) were produced by intravenous injections into rabbits

Two blood group anti A sera were used One was a human isoagglutinin serum kindly supplied by Dr *K Halvorsen* Blood Group Laboratory University Clinic Bergen The other was a rabbit immune serum (R94) produced by intravenous injections of A_1 erythrocytes

The blood group anti O rabbit immune serum (R1084) was produced by intraperitoneal injections

The mononucleosis serum was a pool of sera from patients suffering from infectious mononucleosis The serum agglutinated sheep erythrocytes to a titre of 1 256 and trypsin treated bovine *erythrocytes to a titre of 1 512*

A rabbit anti chicken erythrocyte serum (R99) was produced by intravenous injections

Host antigen This was purified from chicken allantoic fluid as described in (6)

Virus haemagglutination (HA) *and haemagglutination inhibition* (HI) The tests were performed *on perspex plates with vaccinia negative chicken erythrocytes* or with guinea pig erythrocytes The agglutination was read by the pattern method and graded from + to + + + The titres were recorded as the last dilution yielding at least a 1 + reaction

Nonspecific serum HA inhibitors were destroyed by RDE treatment (1 volume of serum and 5 *volumes* of RDE overnight at 37 C) or by treatment with 2 volumes M/60 potassium periodate for 15 min at room temperature followed by a half of glycerol After treatment the sera were heat inactivated at 56 C Normal serum

agglutinins were removed by absorption with chicken or guinea pig erythrocytes in the cold

Haemagglutination inhibition blocking (HIB) This test was performed as described in (3)

HA and haemolysis HA and haemolysis were performed in tubes with heat inactivated anti erythrocyte anti Forssman and mononucleosis sera The sera were diluted twofold serially and a 1 per cent suspension of erythrocytes was added 0 2 ml of each After 30 min at room temperature the erythrocytes were spun down (Adams s serofuge) at 1 000 × g for 30 sec The agglutination was read after re suspension of the erythrocytes by careful tapping of the tubes and was graded from + to +++ Thereafter 0 2 ml of fresh guinea pig serum diluted 1 20 was added to each tube and the haemolysis read after incubation at 37 C for 30 min The HA and haemolysis titres express the highest *dilution of serum (before addition of* the other reagents) giving at least 1 + HA and complete or almost complete haemolysis respectively

Double diffusion in agar was performed by the *Ouchterlony* (18) method

EXPERIMENTS AND RESULTS

Tests for Serological Cross Reactivity between the Host Antigen and Forssman Blood Group and Mononucleosis Antigens

Apart from the presence of sulphate ester groups in the host antigen its chemical composition shows a great resemblance to that of human blood group substances (7) A series of absorption experiments was performed to test for serological cross reactivity

Four HA doses of the following sera were mixed with equal volumes of 0 1 mg per ml of host antigen the two anti Forssman and anti A sera the anti 0 serum the anti chicken erythrocyte serum the anti PR8 allantois sera and the *mononucleosis* serum The anti Forssman anti A and anti PR8 allantoic sera were tested with sheep and human A_1 erythrocytes the mononucleosis serum with sheep and trypsin treated bovine erythrocytes the anti 0 and the anti chicken erythrocyte sera with human 0 and RDE treated chicken erythrocytes No reduction of the HA titres was obtained

The same sera were examined by double diffusion in agar against the purified host antigen No precipitation line could be demonstrated As reported earlier (6) the antiserum to the host antigen gives two precipitation lines with the host antigen

Immunization of rabbits with the host antigen in Freund s complete adjuvant yielded potent HI and precipitating antibodies against the host antigen but no antibodies to the Forssman human blood group A B and O or to the mononucleosis antigens in sheep and trypsin treated bovine erythrocytes (cf Table 1 and 2)

The antiserum to the host antigen was absorbed with the following erythrocytes sheep human A B and O chicken RDE treated chicken and guinea pig Four HI doses of the serum were used and packed erythrocytes were added to a concentration of 20 per cent After 30 min in the cold the erythrocytes were removed by centrifugation and the serum was then re-examined for HI No absorption of HI antibodies could be demonstrated

Thus all these tests failed to demonstrate any serological cross reactivity between the host antigen and the above mentioned substances examined

Tests for Serological Cross Reactivity between Influenza Virus and Forssman, Blood Group and Mononucleosis Antigens

The purity of the virus with regard to allantoic fluid contaminations was checked by titration of host antigen in the HIB test The HIB titre was 1 256 in the original allantoic fluid while the final virus preparation contained no extraneous host antigen The host antigen is easily soluble in water and cannot be adsorbed to erythrocytes In the course of the purification and concentration procedures the HA titre was raised from 1 512 to 1 6 400 When the specific HA activity is calculated relative to allantoic fluid contaminations a purification factor of $\geqq$3 000 was obtained Other impurities might of course have been added from the guinea pig erythrocytes but these will not affect the present serological tests

Several rabbit immune sera produced against allantoically grown influenza viruses were examined by the HA and haemolysis tests with sheep and human A_1 erythrocytes All of them showed a rise in HA and haemolysis with sheep erythrocytes

These anti sheep erythrocyte antibodies could be removed by absorption with a boiled guinea pig kidney homogenate indicating that they are Forssman antibodies

A parallel but less pronounced increase was obtained of anti A agglutinins which also could be removed by absorption with guinea pig kidney Most rabbit preimmune sera contained iso agglutinins to A_1 erythrocytes in titres up to 1 64

Immunization of rabbits with influenza virus purified by adsorption elution from chicken erythrocytes also resulted in a rise in the HA and haemolysis titre with sheep erythrocytes

Immunization of rabbits with the influenza virus preparation which had been obtained by adsorption elution from guinea pig erythrocytes gave no rise in HA and haemolysis titres The same antisera contained potent antibodies to the viral haemagglutinin and to the host antigen component of the haemagglutinin The latter antibodies were identified by means of double diffusion in agar (6) and by their heterotypic HI reactivity The slight rise observed in the haemolysis titre against sheep erythrocytes could be removed by absorption with guinea pig erythrocytes Representative HA and haemolysis data of some of the sera are presented in Table 1

Another approach to this problem was made by examination of different anti Forssman sera in the HI test with purified virus Guinea pig erythrocytes were used for absorption of the sera and for agglutination in the HI test Four different anti Forssman sera (R76 R77 R92,

and R826) were tested for HI activity against 4 HA doses of the virus The sera were treated with potassium periodate to remove unspecific inhibitors since the *Vibrio cholera* filtrate (RDE) was found to contain some Forssman antigen The two anti PR8 allantois sera were absorbed with the purified host antigen before being tested against the heterotypic B Lee strain The other sera were tested against both A and B viruses (Table 2) No HI was produced with serum dilution 1 4 which was the final dilution of serum after treatment with periodate

TABLE 1

Reactivity of Sheep and Human A, Erythrocytes with Antisera to Influenza Virus and Chicken Host Components

Antisera against	Sheep erythrocytes		A erythrocytes
	HA	Haemolysis	HA
A PR8 allantois (R76)	256	4096	64
B Lee allantois (R57)			16
A PR8 chicken eluate (R[illegible]5)	16	512	8
B Lee guinea pig eluate (R85)	<8	<8	8
Allantoic fluid (R87)	<8	32	16
Host antigen (R81)	<8	<8	16

The figures give reciprocals of serum dilutions
The same titre as in the pre immune serum

TABLE 2

HI With Antisera Against Influenza Virus and Host Components Before and After Absorption With Host Antigen

Serum	HI with	
	B Lee	A PR8
Anti A-PR8 allantois unabsorbed (R76)	1024	2048
Anti A PR8 allantois abs with host antigen	<4	2048
Anti B Lee allantois unabsorbed	512	512
Anti B-Lee allantois abs with host antigen	512	<4
Anti normal allantois unabsorbed	1024	512
Anti normal allantois abs with host antigen	<4	<4
Anti host antigen unabsorbed	1024	256

The figures give reciprocals of serum dilutions

The blood group anti A immune serum the anti O serum and the anti-chicken erythrocyte serum were examined for virus HI antibodies in the same way These sera also failed to give an HI The mononucleosis serum gave an HI titre of 1 24 with both the B Lee and the A PR8 virus The HI antibodies could not be removed by absorption with sheep or trypsin treated bovine erythrocytes and are probably virus specific

Absorption of Anti Forssman Anti A, Mononucleosis and Anti Chicken Erythrocyte Sera with Influenza Virus

Four HA doses of the anti Forssman sera R76 R77 R825 and R826 were absorbed with purified virus which had been pre heated at 90 C for 30 min Similar absorptions were carried out with the human iso agglutinin and rabbit anti A sera the anti chicken erythrocyte serum and the mononucleosis serum After absorption the sera were examined in the HA test with the respective erythrocytes No reduction in titre was demonstrated all sera still contained 4 HA doses

DISCUSSION

The influenza virus preparation used in the present experiments had been purified by adsorption to and elution from guinea pig erythrocytes In contrast to chicken erythrocytes guinea pig erythrocytes contain no Forssman blood group A, or mononucleosis antigens

We were not able to demonstrate Forssman blood group A or mononucleosis substances in our virus preparations by means of the HI reaction It has been assumed (19 21 22) that these substances are integral parts of the virus and present at the surface of the virus If this be the case they must occur in very small quantities in the virus compared to the viral and the host antigens The latter antigens react strongly in the HI test while high titred Forssman antisera failed to give any HI

Studies are in progress to see whether degradation of the host antigen will display hidden antigenic sites which may react with antibody to some host tissue material

The same negative results were obtained in the antibody inhibition experiments Our dose of virus was lower than that used by *Rott et al* (19) but comparable to the dose *Springer & Schuster* used (21) When however our virus preparation was used for inhibition of antibodies to the host antigen it was able to neutralize more than 1 000 HI antibody units These experiments also lead to the conclusion that the Forssman and blood group substances if present must occur in very small quantities

The investigations by *Rott et al* (19) showed that serologically active glycolipids of influenza virus preparations such as Forssman blood group A and mononucleosis substances derive from the propagating host cell Highly purified and concentrated virus was used in their experiments It remains however to be shown whether their preparations were completely free of extraneous antigenic material In the case of parainfluenza virus convincing evidence of the acquisition of blood group B substance from Rhesus monkey kidney cells was presented by *Isacson & Koch* (10) Their virus also reacted strongly in the HI test with blood group anti B sera After transfer of the virus to another host virus lost its blood group B activity

As long as the above mentioned host tissue components have not been demonstrated by means of tests which are strictly dependent on a viral activity such as the HI and infectivity neutralization tests no proof has been provided that they are integral parts of the virus or even that they are intimately associated with the virus In the present experiments no HI activity was displayed by high titred Forssman and blood group A antisera Although very sensitive the HI test has certain limitations Firstly it entails a competition for virus between antibody and erythrocyte Secondly the antigenic groupings on the virus surface must be spaced sufficiently near each other if the respective antibodies are to cause steric hindrance of HA reacting sites The first factor cannot be ruled out It is however not likely that the above mentioned antigen antibody systems are of particularly low avidity Regarding the second question anti Forssman and mononucleosis antibodies are found partly or mainly among the γ M globulins which are very active HI antibodies From the molecular size of influenza virus and γ M globulins it is evident that rather few γ M globulin molecules are needed to cover the viral surface

The host antigen has unequivocally been shown to be part of the virus haemagglutinin (9 14) It has the same monosaccharide and amino acid components as human blood group substances but no serological cross reactivity could be demonstrated in the present experiments

We can at least conclude that the host antigen is the only serologically active host component which can be demonstrated by tests which are dependent on intact virus or viral haemagglutinin

The immunization results were also important These showed that removal of allantoic fluid impurities had no influence on the antibody response to the viral haemagglutinin and the host antigen no antibodies being produced against the Forssman human blood group A and mononucleosis antigens

The host antigen is strongly acidic owing to its sulphate half ester groups Polyanionic substances show great affinity to lipoproteins The host antigen is present in those cells of the chorioallantoic membrane that propagate the influenza virus as shown in the tissue culture experiments (8) Other experiments with fowl plague virus (5) indicate that the host antigen is incorporated into the virus only when it is grown in the entodermal layer of the chorioallantoic membrane This inner layer shows high phagocytic activity and has most likely taken up the host antigen from the allantoic fluid It was shown by histochemical methods that this layer is rich in mucopolysaccharide material Probably the host antigen is built into the viral coat when the lipoprotein subunits are assembled near the surface of the cell The strong anionic character of the host antigen makes such an explanation acceptable

No investigations have been made in the present study with regard

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As long as the above mentioned host tissue components have not been demonstrated by means of tests which are strictly dependent on a viral activity such as the HI and infectivity neutralization tests no proof has been provided that they are integral parts of the virus or even that they are intimately associated with the virus In the present experiments no HI activity was displayed by high titred Forssman and blood group A antisera Although very sensitive the HI test has certain limitations Firstly it entails a competition for virus between antibody and erythrocyte Secondly the antigenic groupings on the virus surface must be spaced sufficiently near each other if the respective antibodies are to cause steric hindrance of HA reacting sites The first factor cannot be ruled out It is however not likely that the above mentioned antigen antibody systems are of particularly low avidity Regarding the second question anti Forssman and mononucleosis antibodies are found partly or mainly among the γ M globulins which are very active HI antibodies From the molecular size of influenza virus and γ M globulins it is evident that rather few γ M globulin molecules are needed to cover the viral surface

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No investigations have been made in the present study with regard

to the presence of the host derived protein assumed to the present in influenza virus It is however evident from our results as has also been pointed out by *Laver & Webster* (15) that after absorption of anti influenza immune sera with the host antigen no heterotypic HI antibody can be demonstrated Also the protein host antigen if present must therefore occur in very small amounts

SUMMARY

In the present experiments no serological cross reactivity could be demonstrated between the host antigen and blood group substances

Immunization with crude allantoically cultured influenza virus led to the production of antibodies to Forssman human blood group A and mononucleosis antigens When the virus was purified by adsorption to and elution from guinea pig erythrocytes no such antibody was produced Potent Forssman and blood group A antisera failed to react with the purified virus in the haemagglutination inhibition test

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BRIEF REPORT

TRANSFER AMYLOIDOSIS *IN VITRO* IRRADIATION OF THE TRANSFERRED SPLEEN CELLS

By *Poul Ranløv*

In previous investigations the possible transfer of an experimentally induced amyloidosis between syngeneic mice with spleen cells from donor mice hyperimmunized with casein was demonstrated (*Werdelin* & *Ranløv* 1966 *Ranløv* & *Werdelin* 1967) From these experiments it appeared that such treated recipients developed spleen amyloidosis within 2–4 days following the cell transfer It further appeared that *radioactively labelled* donor cells first and foremost did colonize the recipient spleens and that an intimate correlation in time of appearance and in topography existed between the labelled donor cells and the resulting spleen amyloidosis in the recipient mice Thus it seemed that the hyperimmunized donor cells played an important role in the pathogenesis of the recipient amyloidosis

In order to assess whether x irradiation of the spleen cell suspension derived from the hyperimmunized donors would influence the subsequent amyloidosis in the recipients the following experiment was designed

Randomized inbred mice of the C3H strain were employed Pre immunization of the donors with 17 daily casein injections and preparation of the spleen cell suspension from the pooled donor spleens were performed as earlier described (*Werdelin & Ranløv* 1966) After adjustment to a final concentration of 200 × 10^6 nucleated spleen cells per ml in Ringer's solution the spleen cell suspension was divided into two equal parts one half of the suspension was subjected to x irradiation generated by a Siemens therapy unit (250 kV 1h 0 6 target distance 50 cm dose rate 42 3 rad per minute) to a total dose of 1 000 rad Irradiated and non irradiated cells were injected intravenously into the respective groups of healthy syngeneic recipients as apparent from the data of Table 1 Each mouse received 100 × 10^6 nucleated donor cells in ml of Ringer's solution Half the recipients [..]e subjected to an additional course of nitrogen mustard administered by 3 in[..]ions of 0 05 mg Erasol® in ½ ml saline on the day of transfer and after 2 and [..]a s All recipient mice were killed 5 days after transfer Tissues were fix d in [..]tral formalin paraffin embedded and sections were cut 5 microns thick Amy[..] [..] was *identified by its fluorescence with thioflavin T in the U V light and by* birefringence with Congo red under crossed polars The degree of recipient spleen [..]loidosis was estimated according to the method described by *Christensen & [..]l* (1959)

[..]rom Table 1 it appears that spleen cells derived from the same pool of pre ens[..] [..]d donor spleens pro ed equally effective in inducing amyloidosis in syngeneic [..]pients regardless of any previous x *irradiation of the transferred cells*

[..]etailed knowledge of the influence of ionizing radiation on the single cell is as not available However it seems well established that one major effect of irra[..]ion on lymphoid tissues is an abolished proliferative capacity while such [..]ital [..]ctions as protein and RNA synthesis may go on for months following irradiation doses averaging several thousand rad (*Puck et al* 1956 *Kaplan* 1966) Human peripheral lymphocytes subjected to extracorporeal *irradiation (8 1900 rad) have* been shown to grow and to undergo transformation in tissue culture as do normal lymphocytes the ability of such treated lymphocytes to incorporate H³ labelled thymidine and cytidine remained unaffected (*Fliedner* 1967) Spleen cells in tissue culture may continue to form antibody after irradiation *in vitro* with 10 000 rad (*Makinodan et al* 1966) and the cutaneous reaction of the Normal Lymphocyte Transfer test was not affected by previous x irradiation *in vitro (51 000 rad) of* the transferred lymphocytes (*Wolf Jürgensen* 196[..])

TABLE 1

Effect on the Amyloidosis Inducing Potency of the Transferred Spleen Cells of Previous in vitro X Irradiation of the Cell Suspension

Group	Treatment of donors with casein	Treatment of cell suspension	Treatment of recipients: Number of recipients	Treatment of recipients: Number of spleen cells injected	Treatment of recipients: Nitrogen mustard	Amyloidosis: Incidence	Amyloidosis: Degree (Range)
I	17 daily injections	1 000 rad	8	100×10^6	0	0/8	0 (0–0)
II	– –	1 000 rad	8	100×10^6	+	8/8	3 6 (? 4)
III	– –	None	7	100×10^6	+	7/7	3 4 (? 4)
IV	– –	None	8	100×10^6	0	0/8	0 (0–0)

The results of the present experiment thus seem to indicate that proliferation within the host of the injected syngeneic casein sensitized donor cells is not necessary for their amyloidosis promoting effect Indirectly these results may be taken in support of a concept of an interaction between a messenger function related to material derived from the donor cells and reticular cells of host origin Such a concept fits in with the results obtained in other experiments performed in this laboratory demonstrating a similar transfer of amyloid disease with sub cellula fractions of spleen cells from casein sensitized donor mice (*Ranlov* 1967) These findings are suggestive of a possible existence of two different cell types operating specifically for each of the two phases in experimental amyloid formation described by *Teilum* (1964)

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BRIEF REPORT

MOON CRATER FORMATION ON THE GLOMERULAR BASEMENT MEMBRANE IN HUMAN AND EXPERIMENTAL DIABETES

By *Ruth Østerby Hansen* and *T Steen Olsen*

During our study of glomerular ultrastructure in human and experimental diabetes we have observed a peculiar formation on the basement membrane

The structure was first observed in *alloxan diabetic rats* (9) where is appeared as a pair of spikes projecting from the epithelial side of the basement membrane (Fig 1) The morphologic appearance of the spikes was quite similar to that of the normal basement membrane whereas a fibrillar structure with increased electron density was present in the basement membrane between adjacent spikes A dense fibrillar structure was also seen in the cytoplasm of the epithelial cell immediately above

Wax models prepared on the basis of serial sections showed that the spikes represented sections through mooncrater like structures (Fig 2) The edge of the walls frequently bent inwards so as to narrow the aperture of the crater In most cases the centre of the crater was either at the same level or at a somewhat lower level than the surrounding basement membrane

Crater formations on the basement membrane were found in 5 of 14 alloxan diabetic rats studied by electron microscopy but in none of 9 control rats Crater formations seemed to be more pronounced and more widespread within the glomeruli with increasing duration of diabetes

Since in rats the lesion seemed to be confined to diabetic animals we made a search for a similar lesion in *diabetic patients* Glomeruli from young patients with a duration of diabetes of 0–5 years were studied Montages each of which represented a total glomerular cross section were prepared from the electron micrographs A similar lesion (Fig 3) was found on the basement membrane in 10 out of 29 glomerular cross sections Study of a few serial sections showed that the lesion in these cases also was a crater formation

However a careful study of glomerular cross sections obtained from young *non diabetic patients* without signs of renal disease (8) also revealed the presence of spikes

When the configuration of paired spikes in rats and human beings were compared spikes in the latter cases were generally found to be more blunted the excavation between two spikes was usually not quite as deep and the electron dense material was not a constant finding On the contrary an electron transparent area was sometimes present within the basement membrane delineated by a double membrane like structure (Fig 3) The morphology of the paired spike-configuration was identical in diabetic and non diabetic patients

The paired spikes occur rarely in human individuals as usually only one such pair is present within a whole glomerular cross section An exception from this was glomeruli from patients with diabetes of 0–5 years duration in which 3 7 pairs per cross section were observed However at the present time it is not possible to conclude that a significant difference exists between non diabetics and recent diabetics (on one hand) and patients with diabetes of a few years duration (on the other)

Discussion

We have not been able to find in the literature any descriptions of crater like formation on the glomerular basement membrane In papers on diabetic glomerular

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Fig 1

Glomerular capillary wall from a rat with alloxan diabetes of 10 months duration Two pairs of spikes are seen to project from the epithelial side of the basement membrane Electron dense fibrils are seen in the basement membrane (arrow) and in the epithelial cell between two adjacent spikes



Fig 2

A simplif ed schematic drawing sho ing crater formation s it was revealed by serial sections

lesions we have observed paired pil onf gurat on n the illustrations in two cases (4 6) and in another rep 1) a lesion is d s ribed (but not shown in the illustrations) which eem to r sp nd to our c ater t rmation Among papers dealing with glomerula le n n arious otl er dise ses w have only been able to find a fe micrograph sh ng the hara teri t c structure (2 3 5) These were from cases of glome ul nephr ti nd lupus nephr t s

We observed m n rate l ke structures in non diabet c as well as in diabetic patients and n l abeti rat

The increa d m unt f ba ement membran material pre ent in diab tic glome rulopathy n t lupu nephrit s an l glomerulonephritis well may be the re sult of o e r d t n (7 9) The mo n c ater format n may be the morpholog c app arance t th l ing d wn of n wly synthesized material If this were so the reaction m t be tran tory and fai ly rapid nce crate s are only ra ely seen in the normal n d l A high incid nce of moon craters afte ome years of dia bete a s g t l b the pre ent find ngs may thus represent a pathological in crease of l nent m mb ran s nthesis



Fig 3

Paired spike configuration on the epithelial side of the basement membrane fr m a patient diabetic for 2 years

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CYSTIC PITUITARY TUMOUR DEVELOPED FROM RATHKE'S CLEFT

By

THORBJORN BERGE and STEN ERIKSSON

Received 19 vi 67

Remnants of Rathke's pouch from which the anterior lobe of the pituitary gland is developed consist of a slit shaped cavity (Rathke's cleft) which can often be demonstrated between the anterior and intermediate part of the pituitary The cavity is lined by epithelium which is usually low but sometimes high cylindric and ciliated Sometimes it also contains goblet cells and often a colloid like substance in its lumen Occasionally a number of small cysts are seen instead of one slit shaped cavity These cysts may though rarely proliferate and produce clinical symptoms Such cases have been described by *Duffy* (1920) *Worster Drought & Dickson* (1927) *Frazier & Alpers* (1934) *Smith & Bucy* (1953) A case is described below in which such proliferation caused symptoms suggesting panhypopituitarism

REPORT OF CASE

A man aged 68 was admitted to the emergency unit of the medical clinic on October 4 1964 Until 1963 he had been working as a newspaper roundsman and still occasionally worked as such. He was unmarried living alone and had no close relatives from whom anamnestic data could be obtained He reported that he had felt well untill October 1 1964 when he fell ill with diarrhoea nausea and chills During the following few days he had mild symptoms of colitis but on the day of admission he had felt much worse and had respiratory distress with air hunger especially when recumbent He denied chest pain He said he had passed only small amounts of urine the previous few days but added that he had also drunk only little during that time He had been upp and about the last few days but had not worked

Clinical Findings

On admission he was found to be in some distress His degree of wakefulness was slightly lowered he showed signs of slight confusion and he could not give a satisfactory history The fingers [illegible] s were cyanotic and cold No peripheral oedema was seen The respiration was of the Kussmaul type Body hair was scanty axillary hair was lacking the pubic hair growth was reduced and of female type. The skin was thin and atrophic and finely wrinkled and there was a pallor out of proportion to the anaemia found

No heart sounds could be heard and the radial pulses were not palpable The blood pressure could not be measured in either arm Fine inspiratory rales were heard over the lung bases The abdomen was soft the liver edge was felt about 4 cm below the right costal arch The prostatic gland was felt soft and small for the patient's age Neurologic examination revealed nothing remarkable except pos



Fig 3

Paired spike configuration on the epithelial side of the basement membrane from a patient diabetic for 2 years

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CYSTIC PITUITARY TUMOUR DEVELOPED FROM RATHKE'S CLEFT

By

Thorbjorn Berge and Sten Eriksson

Received 19 vi 67

Remnants of Rathke s pouch from which the anterior lobe of the pituitary gland is developed consist of a slit shaped cavity (Rathke s cleft) which can often be demonstrated between the anterior and intermediate part of the pituitary The cavity is lined by epithelium which is usually low but sometimes high cylindric and ciliated Sometimes it also contains goblet cells and often a colloid like substance in its lumen Occasionally a number of small cysts are seen instead of one slit shaped cavity These cysts may though rarely proliferate and produce clinical symptoms Such cases have been described by *Duffy* (1920) *Worster Drought & Dickson* (1927) *Frazier & Alpers* (1934) *Smith & Bucy* (1953) A case is described below in which such proliferation caused symptoms suggesting panhypopituitarism

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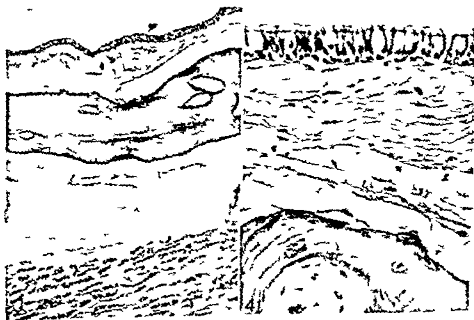
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reduction of the renal parenchyma At the transition between the related renal pelvis and the ureter which was of normal width was an occluding calculus The prostate was if anything somewhat small with a grey white moist cut surface The coronary arteries were atheromatous In addition the *descending branch of the left coronary vessel* was occluded 2 cm from its origin by a recent thrombus In the myocardium *the ventricular septum and adjacent parts of the anterior* wall of the left ventricle showed recent infarction with a haemorrhagic margin *In addition a fibrous scar 2 cm in diameter was seen in the* lateral part of the posterior wall

There were general signs of cardiac insufficiency

HISTOLOGIC EXAMINATION

The tumour at the site of the hypophysis was made up of bony tissue which was *partly necrotic It consisted mainly of fine trabeculae with* loose connective tissue in the marrow spaces enclosing numerous large *and small cysts The inner wall of these cysts was lined by simple cy*lindric ciliated epithelium Goblet cells were seen in several areas No structures *resembling adamantinoma were seen Some sections from* different part of the tumour showed peripherally small streaks of pre



Figs 3–4

Fig 3 Cyst with cylindrical epithelium on loose connective tissue and with regular bony tissue in the wall At bottom of picture streaks of preserved adenohypophyseal tissue (*H E* × 75)

Fig 4 Ciliated cylindrical epithelium (H E × 380)

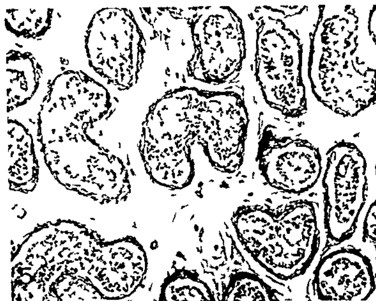


Fig 5

Testes with peritubular fibrosis and complete lack of Leydig cells (H E. × 80)

served adenohypophyseal tissue with apparently normal proportions between the various types of cells (Figs 3 and 4) Neurohypophyseal tissue was not seen

The testes contained sparse tubuli mostly with Sertoli cells Scattered tubules showed signs of spermiogenesis Not only spermatogonia but also a few spermatozoa were seen There was marked peritubular fibrosis The other interstitial tissue showed relatively mild fibrosis No Leydig cells were found (Fig 5)

The thyroid contained medium sized follicles with readily staining eosinophilic colloid in the lumina The epithelium was as a rule low cuboidal

The adrenals showed no remarkable histologic changes The pancreas was not examined histologically The right kidney showed severe parenchymal atrophy On the left side there was moderate pronounced arterio and arteriosclerotic changes

The prostate contained regular glandules with low epithelium showing no signs of malignancy

DISCUSSION

The clinical findings that aroused the suspicion of panhypopituarism were above all those seen on external examination sparse growth of body hair lack of axillary hair growth feminine outline of pubic hair pale skin and relative prostatic atrophy all signs of hypogonadism with severe androgenic deficiency

produced a clinical picture of dyspituitarism of Lorain Lewi type The symptoms in these cases did not differ from those of other pituitary tumours In these patients surgery can also be successful (*Frazier & Alpers* (1934) *Smith & Bucy* (1953))

SUMMARY

A case of cystic hypophyseal tumour developing from remnants of Rathke s cleft in a 68 year old man is described The tumour which was the size of a tangerine was built up of multiple pea sized cysts lined by a regular ciliated cylindrical epithelium The periphery of the tumour contained small streaks of preserved adenohypophyseal tissue

The patient showed the picture of panhypopituitarism Post mortem findings and laboratory data corroborated the occurrence of secondary hypogonadism affecting interstitial cell function and to a large extent also spermiogenesis A differential failure of FSH and ICSH activity was probably present

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SENSITIVITY AND REPRODUCIBILITY OF A MICROPLATE TECHNIQUE FOR IMMUNODIFFUSION

By

ULF KRAUSE and VEIJO RAUNIO

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In analyses of immune precipitates in gel a slide microtechnique described by *Wadsworth* (1957) has been found of great value owing to its sensitivity and the short time required for obtaining registrable immune precipitates.

The reproducibility of this technique, however, is not quite satisfactory and should as far as possible be improved. It often happens that the whole diffusion procedure fails because of leakage of reactants between the surface of the plexiglass cover and the agar layer. This leakage in turn may be due to the fact that the gel shrinks upon congelification and the contact between agar and plexiglass surfaces is lost. Difficulties have also been experienced in achieving a uniform shape and size of the agar basins at the bottom of plexiglass holes.

In this investigation the above disadvantages have been considered and some tricks introduced to improve the method. The sensitivity of the method thus modified has also been tested.

METHODS

Plexiglass slides were prepared according to the directions of *Wadsworth*. To provide a chamber of the desired depth (0.4–0.6 mm) we used thin glass plates (microscopical cover glasses) instead of the waterproof tape strips suggested by *Wadsworth*. Four cover glasses were placed on an agar filmed slide to form the pattern shown in Fig. 1. The plexiglass matrix was then lowered into place in such a way that it was supported by a corner of each cover glass. Instead of cover glasses also thin nylon threads could be used. Before the pouring of agar, a small stainless steel bead was placed in each hole of matrix. The size of the steel beads (Ø 2.36 mm) was so chosen that the latter closed the lower aperture of the hole (Ø 2 mm) tightly. The purpose of these beads is to facilitate the moulding of uniform agar basins at the bottom of the holes. They also prevent drying of the gel at the basin apertures.

A sufficient amount of 1 per cent agar (Difco purified agar) in 0.9 per cent NaCl of 50–60° was allowed to flow into the chamber from one of the open sides. If in the process of pouring the plate, the agar is forced up into the holes, it can be removed at the same time as the steel beads by vacuum after gelification.

It is important that the pouring is performed after the base slide and matrix have been prewarmed and placed on a warm surface. Immediately after pouring, the plate is set aside to cool. After about 30 min. the cover glasses are removed two at a time

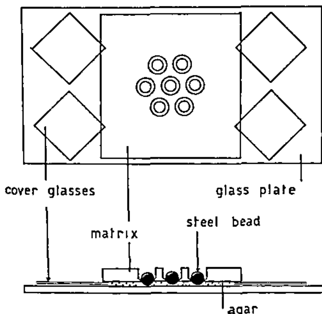


Fig 1

Drawing showing a gel chamber formed by a plexiglass matrix steel beads micro scopic cover glasses and glass slide After gelification of agar steel beads and cover glasses are removed

by carefully pulling out the opposite cover glasses This is accomplished more readily if only a very small part of the cover glass corner lies between matrix and base slide

After this procedure the plexiglass matrix rests entirely on the agar and thus a tight contact between the agar layer and the lo er surface of matrix is achieved The steel beads are not removed until just before the holes are charged with reac tants (5 to 10 microlitres)

The precipitates were allowed to develop for three days After careful removal of the plexiglass covers the slides were washed in buffered (pH 7) saline for two days They were then dried and stained with Amido Black in the usual manner

To achieve optimal resolution and crispness of pre ipation lines in the gel the effect of some physical factors such as pH salt concentration temperature and thickness of the agar layer were also tested one at a time keeping other conditions constant For the same reasons each antigen dilution was tested against several antibody dilutions ranging progressively from $\frac{1}{1}$ to $\frac{1}{3}$ in two fold steps For com parison macro diffusion plates were prepar d and tested by using same antigens as in the tests of microplates

The distance of the basins in the ma ro Ouchter' lates was 4 mm A p ttern with circumferentially arranged r d basins was employed

IATERIAL

Pur fie l ant gens—The follo n purified antig ns were u ed

1) Hum n albumin trock reinst" suppl ed b B hr ngwerke Marburg Lahn
2) Human transferrin B I ngs erke
3) Human gamma globul n 12 per cent solution ol t ine l from AB Kab Stock holm Sweden

Stock solutions containing 10 mg% of protein were made For sensitivity tests a

with a total of 0.015 to 0.03 μg of antigen detected in the microplates. This result, even though not quite comparable to the data in *Kabat's* book because we used heterologous antigens, suggest an about equal level of sensitivity with the Oudin system.

Similar sensitivity is indicated by the results obtained when antigens in total human serum were tested by specific antisera. However, as far as the amount of antigen detectable is concerned, there is wide variation depending on the antigen-antibody system studied.

The reproducibility of *Wadsworth's* microtechnique is discussed also by *Holm* (1965) and improvements suggested. Holm's ideas of using Petri dishes instead of glass slides and agar in excess should be adaptable to our modification. This combination might further enhance the reproducibility.

SUMMARY

A modification of microplate technique for immunodiffusion is described and its reproducibility tested. For immunodiffusion procedures a thin gel chamber was formed by a plexiglass matrix, steel beads, microscopic cover glasses and a glass slide. Good reproducibility was achieved. Estimation of the sensitivity of microplates showed that as best a total amount of 0.015 to 0.03 μg of antigen was needed to disclose visible precipitates.

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IDENTIFICATION OF MOULDS BY SEROLOGIC DIFFERENTIATION OF THEIR PROTEOLYTIC ENZYMES

By

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Received 11 iv 67

Serologic examination of mould antigens has been carried out with various genera including *Aspergillus* and *Penicillium* (*Seeliger* 1958 *Pepys et al* 1959 *Longbottom et al* 1960 *Biguet et al* 1964 *Longbottom & Pepys* 1964) Most of the works deal with somatic antigens of polysaccharide or polysaccharide protein nature examined by precipitation tests mainly agar gel diffusion Various other methods have also been tried (*Seeliger* 1958) So far fluorescent antibody technique has not been extensively used but *Schmidt & Bankole* (1962) have used the method for detection of *Aspergillus* hyphae in soil

There is a great deal of confusion with regard to the specificity of mould antigens (*Longbottom et al* 1960) but many of the antigen complexes seem to include components with a tendency to overlap the borders of species and genera At present serologic tests with somatic antigens do not seem to be particularly useful in routine classification of moulds probably because it is difficult to select antigens representing specific criteria of genus and species

In the last decade extracellular antigens have been of interest as basis for taxonomic differentiation of bacteria (*Liu* 1961 a b and 1962 *Sandvik* 1962 *Sandvik & Fossum* 1965) Thus serologic classification of bacterial enzymes by means of specific antienzymes has been used to identify the corresponding organisms The principle is similar to the well known identification of exotoxin producing organisms by means of specific antitoxins The last mentioned authors consider serologic identification of biocatalysts to be a most valuable taxonomic criterion for bacterial species

Serologic examination of extracellular proteinases of bacteria by means of an immunoelectrophoretic method has been described (*Sandvik* 1962) By this method specific antiproteinases in immune rabbit sera were separated from normal proteinase inhibitors by paper electrophoresis after which their antiproteolytic effect was demonstrated by inhibiting the specific enzyme from precipitating sodium caseinate in an agar medium It was shown that many serologically

different proteolytic enzymes were produced by the organisms examined. Most of the enzymes were specific in respect of species and cross reactions between species and genera were exceptional. Thus using suitable antigens which are labeled by their functional enzymatic properties it is possible to identify various organisms even though the enzymes are not purified.

The said method has been used in the present investigations to examine proteolytic moulds of the genera *Aspergillus*, *Penicillium* and *Scopulariopsis*.

MATERIALS AND METHODS

Strains.—Nineteen strains of the genera *Aspergillus*, *Penicillium* and *Scopulariopsis* used were type cultures obtained from American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. They are all listed in Table 1. One strain of *Aspergillus flavus* (A3734/10) was a gift from Mr. P. K. C. Austwick, the Central Veterinary Laboratory, Weybridge, England, to the Department of Food Hygiene, Veterinary College of Norway. Over longer periods the cultures were stored at −25 C on Sabouraud's Dextrose Agar (Difco) or freeze dried.

Enzymes.—The various proteinases were produced by growing the organisms on the surface of semi solid skim milk agar (nutrient broth 40 per cent, nutrient agar 4? per cent, autoclaved skim milk 28 per cent) in Roux bottles for 4–6 days at room temperature. Heavy nutrient broth suspensions of cells from cultures on Sabouraud's agar were used for inoculating the Roux bottles. Harvesting of cultures and enzyme concentration and purification procedures have been described (Sandvik, 1962). Briefly, agar cultures were frozen and the liquid phase harvested after thawing. The liquid was centrifuged, ammonium sulphate added to the supernatant fluid to 80 per cent saturation and the mixture kept at 4 C overnight. The precipitate was separated by centrifugation or filtration, resuspended in a small volume of distilled water, and dialysed with constant agitation against distilled water at 5 to 8 C. Sometimes the procedure was repeated once or twice with decreasing concentrations of ammonium sulphate (down to 60 per cent saturation). For development of the immunoelectrophoretic patterns (see later) unconcentrated culture supernatant or filtrate can also be used.

Sera.—As a rule, antiproteinases were produced in rabbits given injections of the crude concentrated enzymes mixed with equal amounts of Freund's complete adjuvant (Difco) (other lipid adjuvants were also used successfully). The mixture was injected subcutaneously into rabbits in amounts of 1.0, 2.0 and 4.0 ml at 6 day intervals. One week after the 3rd injection the blood sera were tested for specific antienzymes. When it was found difficult to obtain a satisfactory titre of an antienzyme, additional injections of 0.5 to 1.0 ml of enzyme preparation without adjuvant were given intravenously.

Caseinate Medium.—The medium used to test for proteinase activity was prepared as follows: agar (Difco Bacto agar 0140–01) 1.40 per cent, sodium caseinate (added as 4.0 per cent solution of pH 6.?) 1.00 per cent, thimerosal 0.01 per cent, $MgCl_2$ (added as 10.0 per cent wv solution) 0.004 M, in distilled water. The pH was adjusted to 6.2. Agar thickness of the plates was ? mm. If correctly prepared the plates will remain clear and transparent after being incubated at 37 C overnight.

Serologic Differentiation.—The antisera were electrophorized by paper electrophoresis before being brought into contact with the enzymes. A type 3276 BN LKB (Stockholm) apparatus was used with Schleicher and Schüll No. 2043 bmgl paper. Usually 0.05 M phosphate buffer, pH 6.2, was used. Thimerosal was added to the buffer to a final concentration of 1:10 000. The sera were applied in 8–10 µl amounts and electrophorized at 1?0 v for 16 to 18 hours. The wet paper strips were transferred immediately to the surface of the caseinate medium. After incubation at 37 C for 2 to 3 hours the strips were removed from the medium and replaced by narrow (0.5 to 0.8 cm) strips of filter paper that had been immersed in solutions containing the proteinases to be tested. The enzyme solutions were preserved by the addition of thimerosal (1:10 000). Three to four filter paper strips could be placed in parallel lines within the 4 cm broad field of the electrophoresis paper on the agar surface

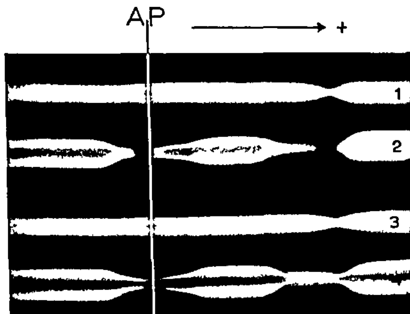


Fig 1

Electrophoretic patterns for antiserum against *Aspergillus flavus* (ATCC 15517) proteinase transferred to sodium caseinate agar Developments are performed with 4 different enzymes one of which (2) is homologous to the antiserum The other enzymes (1 3 and 4) are produced by *Aspergillus fumigatus* (ATCC 1028) *Penicillium roqueforti* (ATCC 6989) and *Aspergillus oryzae* (ATCC 9362) respectively The specific antibodies are localized in the area of the line of application (AP) and the normal serum inhibitors to the right of this line The electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 hours at 120 V

The enzyme containing strips were removed after 4 to 18 hours at 37 C depending on the amount of development desired Precipitation zones occurred along the enzyme-containing strips (Fig 1) As shown in the previous report (*Sandvik* 1962) in certain places the zones were interrupted by zones in which precipitation was absent as a result of inhibition of the proteolytic enzymes The normal inhibitors in serum were localized in the α and β globulins on the anode side of the line of application but the inhibition due to specific antienzymes occurred in the area of the γ globulins which under the present conditions were situated at or near the line of application (Fig 1)

RESULTS

Strains of the species *Aspergillus flavus* *Asp oryzae* *Asp clavatus* *Asp fumigatus* and *Asp tamarii* produced considerable amounts of proteolytic enzymes (Casein Precipitating Enzymes or CP enzymes *Sandvik* 1962) Immunization carried out with *Asp flavus* (ATCC 15517) and *Asp fumigatus* (ATCC 1028) resulted in high yields of antienzymes *Asp niger* (ATCC 6275) was a poor proteinase producer although sufficient to enable the immunoelectrophoretic test to be carried out with unconcentrated culture supernatant An attempt to induce antienzyme production in rabbit with concentrated *Asp niger*

CP enzymes serologically unrelated to those of *Scopulariopsis brevicaulis* (ATCC 7123) and *Penicillium notatum* (ATCC 9178). Serologic subdivision of these species seems therefore to be possible by the method used.

It is important that the term *antienzyme* in the present paper should be taken to mean serum inhibitors of γ globulin nature specifically inhibiting the biocatalytic activity of the enzymes. A corresponding specificity may not be the case for other serologic reactions (gel precipitation for example) between enzyme and antienzyme.

SUMMARY

Proteolytic enzymes produced by 20 strains of the genera *Aspergillus*, *Scopulariopsis* and *Penicillium* have been serologically compared. The technique is a special immunoelectrophoretic procedure in which the proteolytic activity of enzymes is neutralized by specific antisera. No intergeneric crossreactions could be demonstrated. Inter species cross reactions occurred between enzymes of some *Aspergillus* species i.e. *Asp flavus*, *Asp oryzae*, *Asp clavatus* and *Asp tamarii* while enzymes of other species examined were serologically unrelated. Within the species *Scopulariopsis brevicaulis* and *Penicillium notatum* two serologically different types were demonstrated.

The possibility of using *enzymo serologic* methods as an aid in classification of moulds has been discussed.

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IMMUNOCHEMISTRY OF *ESCHERICHIA COLI* O ANTIGENS

By

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and O WESTPHAL

Received 2 v 67

In a series of papers *Kauffmann Westphal, Luderitz* and co workers (17 18 19 20) reported on the analysis of the sugar constituents of cell wall lipopolysaccharides—*O antigens*—of a great number of different enterobacterial strains Most extended was the examination of more than a hundred selected *Salmonella* strains and the correlation of *serotypes* on the basis of the *Kauffmann White* scheme and the sugar composition of their lipopolysaccharides (*chemotypes*) (20) These authors also performed some preliminary studies on *Escherichia coli* (17) and various other Enterobacteriaceae groups especially serotypes exerting serological cross reactions with *Salmonella* strains (18 34) because of structural relationships of their respective O antigens (common or partly common O factors) The 29 *E coli* strains analysed (17) consisted of type strains for the most common serotypes isolated from infantile diarrhoea further a number of strains from normal faeces some of them O antigenic tests strais some freshly isolated strains and finally some few strains isolated from various non enteric pathological conditions

So far about 150 *E coli* O antigenic test strains are known In a more extended comparative investigation during the recent years we have analysed about hundred *E coli* O antigens (lipopolysaccharides) Almost all cultures were O antigenic test strains Analysis of the remaining about 50 O antigenic test strains is in progress The present paper describes the sugar composition of the analysed *E coli* O antigens and their classification into known and new chemotypes Some comparative aspects of the chemistry immunochemistry and biology of Enterobacteriaceae O antigens will be discussed on the basis of our result

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[1] In part extract from Thesis by *B Jann* University of Freiburg 1965 (8)
[2] In part extract from Thesis by *F Müller Seitz* University of Freiburg 1967 (10)

TABLE 1 (cont.)

Chemotype	Strain Number	Serotype O	K	H	3 Amino 3 6 dideoxy glucose	3 Amino 3 6 dideoxy D galactose	2 Amino 2 6 dideoxy mannose	2 Amino 2 6 dideoxy galactose	Galactosamine	Glucosamine	2 keto 3 deoxyoctonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6 Deoxy talose	Colitose	Ribose
III	G 3404/41	8	8	4						+	+	+	+	+	+					
	Bi 316/42	9	9	12						+	+	+	+	+	+					
	H 316	40	?	4						+	+	+	+	+	+					
	F 8902/41	58	?	27						+	+	+	+	+	+					
	H 308b	*93*	*?*	–						+	+	+	+	+	+					
IV	Bi 7458/41	6	2ac	1					+	+	+	+	+	+	+					
V	H 710c	41	?	40						+	+	+	+	+		+				
	U 20/41	52	?	10						+	+	+	+	+		+				
VI	F 71	80	?	26					+	+	+	+	+	+		+				
	H 35	86	?	2a					+	+	+	+	+	+		+				
	H 77	90	?	–					+	+	+	+	+	+		+				
	Cigleris	128	67	2					+	+	+	+	+	+		+				
VII	U 5/41	1	1	7						+	+	+	+	+			+			
	Su 4321/41	13	11	11						+	+	+	+	+			+			
	F 10018/41	18	76	14						+	+	+	+	+			+			
	F 8188/41	19ab	–	7						+	+	+	+	+			+			
	F 77a	35	?	10						+	+	+	+	+			+			
	H 7	39	?	–						+	+	+	+	+			+			
	U 18/41	50	?	4						+	+	+	+	+			+			
	Bi 7327/41	53	?	3						+	+	+	+	+			+			
	Su 3972	54	?	2						+	+	+	+	+			+			
	F 10167a/41	60	?	33						+	+	+	+	+			+			
	P 9b	69	?	38						+	+	+	+	+			+			
	H 504c	99	?	33						+	+	+	+	+			+			
	H 509a	100	?	2						+	+	+	+	+			+			
	H 511	102	?	8						+	+	+	+	+			+			
	178/54	129	?	11						+	+	+	+	+			+			
VIII	U 8/41	48	?	–					+	+	+	+	+	+			+			
	U 12/41	49	?	12					+	+	+	+	+	+			+			
	U 19/41	51	?	24					+	+	+	+	+	+			+			
	30 w	117	?	4					+	+	+	+	+	+			+			
X	Stoke W	111	58	–						+	+	+	+	+					+	
XI	Su 3912/41	55	59	–					+	+	+	+	+	+					+	
XII	Bi 623/41	11	10	10					+	+	+	+	+	+	+	+				
	Bi 7455/41	43	?	2					+	+	+	+	+	+	+	+				

TABLE 1 (cont.)

Chemo type	Strain Number	Serotype O	K	H	3 Amino 3 6 dideoxy glucose	3 Amino 3 6 dideoxy D galactose	2 Amino 2 6 dideoxy mannose	2 Amino 2 6 dideoxy galactose	Galactosamine	Glucosamine	2 keto 3 deoxyoctonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6 Deoxy talose	Colitose	Ribose
XVII	H 304	34	?	10						+	+	+	+	+	+		+			
	P 7d	68	?	4						+	+	+	+	+	+		+			
	F 3b	75	?	5						+	+	+	+	+	+		+			
	E 49	79	?	40						+	+	+	+	+	+		+			
XVIII	H 70 c	44	74	18						+	+	+		+	+					
	F 909a	59	2	19						+	+	+		+	+					
	P 12a	73	2	31						+	+	+		+	+					
	F 10	77	?	–						+	+	+		+	+					
XIX	K 12a	17	16	18						+	+	+		+	+		+			
XX	Bi 626/42	12	5	4				+		+	+	+	+	+						
	F 7902/41	15	14	–				+		+	+	+	+	+						
	Su 4338/41	29	?	10				+		+	+	+	+	+						
	F 8198/41	57	?	–				+		+	+	+	+	+						
XXI	U 4/41	4	3	5				+		+	+	+	+	+			+			
	F 11119/41	16	1	–				+		+	+	+	+	+			+			
	E 47a	25	19	12				+		+	+	+	+	+			+			
	H 311b	26	60	–				+		+	+	+	+	+			+			
XXII	H 61	45	?	10				+		+	+	+	+	+				+		
XXIII	U 14/41	3	2ab	2			+			+	+	+	+	+						
XXIV	H 502a	36	?	9						+	+	+	+	+	+	+	+			
XXVI	P 1a	66	?	25						+	+	+	+	+	+			+		
	H 53	88	?	25						+	+	+	+	+	+			+		
XXVII	H 19	84	?	21						+	+	+	+	+		+		+		
XXVIII	F 3a	74	?	39		+				+	+	+	+	+						
XXIX	U 9/41	2	1	4		+				+	+	+	+	+			+			
XXX	U 1/41	5	4	4	+				+	+	+	+	+	+						
	K 11a	65		–	+				+	+	+	+	+	+						
XXXI	P 9c	70	?	42	+				+	+	+	+	+	+		+				
XXXII	P 10a	71	?	12	+				+	+	+	+	+	+			+			
XXXIII	26w	114	90	32	+					+	+	+	+	+						-

TABLE 1 (cont.)

Chemotype	Strain Number	Serotype O	K	H	3 Amino 3 6 dideoxy glucose	3 Amino 3 6 dideoxy D galactose	2 Amino 2 6 dideoxy mannose	2 Amino 2 6 dideoxy galactose	Galactosamine	Glucosamine	2 keto 3 deoxyoctonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6 Deoxy talose	Colitose	Ribose
III	G 3404/41	8	8	4						+	+	+	+	+	+					
	Bi 316/42	9	9	12						+	+	+	+	+	+					
	H 316	40	?	4						+	+	+	+	+	+					
	F 8962/41	58	?	27						+	+	+	+	+	+					
	H 308b	*93*	*?*	–						+	+	+	+	+	+					
IV	Bi 7458/41	6	2ac	1					+	+	+	+	+	+	+					
V	H 710c	41	?	40						+	+	+	+	+		+				
	U 20/41	52	?	10						+	+	+	+	+		+				
VI	E 71	80	?	26					+	+	+	+	+	+		+				
	H 35	86	?	25					+	+	+	+	+	+		+				
	H 77	90	?	–					+	+	+	+	+	+		+				
	Cigleris	123	67	2					+	+	+	+	+	+		+				
VII	U 5/41	1	1	7						+	+	+	+	+			+			
	Su 4321/41	13	11	11						+	+	+	+	+			+			
	F 10018/41	18	76	14						+	+	+	+	+			+			
	F 8188/41	19ab	–	7						+	+	+	+	+			+			
	E 77a	35	?	10						+	+	+	+	+			+			
	H 7	39	?	–						+	+	+	+	+			+			
	U 18/41	50	?	4						+	+	+	+	+			+			
	Bi 7327/41	53	?	3						+	+	+	+	+			+			
	Su 3972	54	?	2						+	+	+	+	+			+			
	F 10167a/41	60	?	33						+	+	+	+	+			+			
	P 9b	69	?	38						+	+	+	+	+			+			
	H 504c	99	?	33						+	+	+	+	+			+			
	H 509a	100	?	2						+	+	+	+	+			+			
	H 511	102	?	8						+	+	+	+	+			+			
	178/54	129	?	11						+	+	+	+	+			+			
VIII	U 8/41	48	?	–					+	+	+	+	+	+			+			
	U 12/41	49	?	12					+	+	+	+	+	+			+			
	U 19/41	51	?	24					+	+	+	+	+	+			+			
	30 w	117	?	4					+	+	+	+	+	+			+			
X	Stoke W	111	58	–						+	+	+	+	+					+	
XI	Su 3912/41	55	59	–					+	+	+	+	+	+					+	
XII	*Bi 623/41*	11	10	10					+	+	+	+	+	+	+	+				
	Bi 7455/41	43	?	2					+	+	+	+	+	+	+	+				

TABLE 1 (cont)

Chemo type	Strain Number	Serotype O	K	H	3 Amino 3 6 dideoxy glucose	3 Amino 3 6 dideoxy D galactose	2 Amino 2 6 dideoxy mannose	2 Amino 2 6 dideoxy galactose	Galactosamine	Glucosamine	2 keto 3 deoxyoctonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6 Deoxy talose	Colitose	Ribose
XIII	H 304	34	?	10						+	+	+	+	+	+		+			
	P 7d	68	?	4						+	+	+	+	+	+		+			
	F 3b	75	?	5						+	+	+	+	+	+		+			
	E 49	79	?	40						+	+	+	+	+	+		+			
XVII	H 702c	44	74	18						+	+	+		+	+					
	F 9095	59	2	19						+	+	+		+	+					
	P 12a	73	2	31						+	+	+		+	+					
	E 10	77	?	–						+	+	+		+	+					
XIX	K 12a	17	16	18						+	+	+		+	+		+			
XX	Bi 626/42	12	5	4				+		+	+	+	+	+						
	F 7902/41	15	14	–				+		+	+	+	+	+						
	Su 4338/41	29	?	10				+		+	+	+	+	+						
	F 8198/41	57	?	–				+		+	+	+	+	+						
XXI	U 4/41	4	3	5				+		+	+	+	+	+			+			
	F 11119/41	16	1	–				+		+	+	+	+	+			+			
	F 47a	25	19	12				+		+	+	+	+	+			+			
	H 311b	26	60	–				+		+	+	+	+	+			+			
XXII	H 61	45	?	10				+		+	+	+	+	+				+		
XXIII	U 14/41	3	2ab	2			+			+	+	+	+	+						
XXIV	H 502a	36	?	9						+	+	+	+	+	+	+	+			
XXVI	P 1a	66	?	25						+	+	+	+	+	+			+		
	H 53	88	?	25						+	+	+	+	+	+			+		
XXVII	H 19	84	?	21						+	+	+	+	+		+		+		
XXVIII	E 3a	74	?	39		+				+	+	+	+	+						
XXIX	U 9/41	2	1	4		+				+	+	+	+	+			+			
XXX	U 1/41	5	4	4	+				+	+	+	+	+	+						
	K 11a	65	?		+				+	+	+	+	+	+						
XXXI	P 9c	70	?	42	+				+	+	+	+	+			+				
XXXII	P 10a	71	?						+	+	+	+	+	+			+			
XXXIII	26 v	114	90	3						–	+	+	+							+

From Table 1 it can be seen that with a few exceptions all lipopoly saccharides—independent of the serological O group—contain 2 keto 3 deoxy octonic acid (KDO) heptose glucosamine galactose and glu cose The same sugar constituents are common to *all* hitherto analysed lipopolysaccharides of *Salmonella* wild type strains (O antigens of S forms) It is known (26) that these sugars (basal sugars) constitute the so called *basal core* of *Salmonella* lipopolysaccharides to which in the O antigens of the S forms long side chains composed of repeating units of species specific oligo saccharides are bound The structure of the side chains is responsible for the serological O specificity (26) whilst the inner core structure composed of basal sugars is responsible for R specificities given by R forms They are mutants with blocks in the biosynthesis of the complete O antigenic (lipo)polysaccharide (26)

Table 1 shows that the 5 basal sugar constituents of Salmonella *O antigens are generally also found in* E coli *O antigens*

In addition to the basal sugars various other sugar constituents can take part in the (specific) structure—*i e* O antigenic side chains— of *E coli* lipopolysaccharides The following sugars were found some of them being discovered for the first time as constituents of entero-bacterial cell wall polysaccharides

Hexose	Mannose
6-deoxyhexose	Fucose Rhamnose 6 deoxy (L) talose
3 6 dideoxy hexose	Colitose (3 6 dideoxy L galactose)
Pentose	Ribose
2 amino 2 deoxyhexose	Galactosamine
2 amino 2 6 dideoxy hexose	Fucosamine Rhamnosamine
3amino 3 6 dideoxy hexose	3 amino 3 6 dideoxy glucose 3 amino 3 6 dideoxy D galactose

Hexoses and 6 deoxyhexoses *Mannose fucose* and *rhamnose* are fre quently found either alone or in combination *6 deoxy talose* was de tected in the lipopolysaccharide of O groups 45 66 84 and 88 To our knowledge this is the first time that 6 deoxy talose has been found in enterobacterial strains In one case (*E coli* 045) the L configuration of 6 deoxy talose has been established by isolation and measurement of its optical rotation (8) Generally enterobacterial lipopolysaccharides were found not to contain more than *one* isomer of the 6 deoxy hexose series However in *E coli* 036 fucose *and* rhamnose and in *E coli* 084 fucose *and* 6 deoxy talose are definitely constituents of the same lipopoly saccharide

3 6 dideoxy hexoses As already described by *Westphal et al* (35)

colitose (3 6 dideoxy L galactose) is a constituent of the *E coli* 055 and *0111* antigens We did not find colitose in additional *E coli* lipopoly saccharides nor has any other dideoxy hexose been detected

Pentoses *Ribose* is known to be a constituent of some *Salmonella O antigens (18 20)* *We found ribose (ca 3 per cent) to be definitely* present in the lipopolysaccharide of *E coli* 0114 The purified prepara tion does not give any absorption at 260 mμ indicating that contamina tion with nucleic acid can be excluded In several other cases namely *E coli* 05 054 081 and 0100 ribose was found in hydrolysates of the purified lipopolysaccharides but in very small amounts Until further studies have given real proof of ribose as a constituent of these lipo polysaccharides they are preliminarily listed in Table 1 as ribose free

2 amino 2 deoxy hexoses *Glucosamine* was found in all lipopoly saccharides investigated Its identification as a constituent of the poly saccharide component of *E coli* lipopolysaccharides would be of im portance because besides its ubiquitous occurrence in the lipid com ponent (lipid A) glucosamine takes part in the inner structure of all *Salmonella* lipopolysaccharides (RII structure see (26)) or else can be part of the specific side chain In the present investigation we did not differentiate between lipid bound and polysaccharide bound glucosa mine Work along these lines is in progress —The presence of *galac tosamine* in *E coli* lipopolysaccharides has already been described (20) During these investigations we detected galactosamine in the lipopoly saccharides of additional *E coli* strains

2 amino 2 6 dideoxy hexoses *Fucosamine* was found in the lipopoly saccharides of 9 different *E coli* strains In the lipopolysaccharides isolated from *E coli* 012 015 029 and 057 fucosamine occurs together with the common basal sugars (chemotype XX) The lipopolysacchar ides of *E coli* 04 016 025 and 026 contain rhamnose in addition whereas the lipopolysaccharide of *E coli* 045 contains 6 deoxy L talose as a further constituent Fucosamine has not yet been described as a constituent of enterobacterial lipopolysaccharides Recently *Barry* (2) *Barry & Roark* (4) isolated fucosamine from a mucopolysaccharide of *Citrobacter* *Salmonella* and *Arizona* There are indications that fuco samine is also a constituent of the O specific lipopolysaccharide of these strains (*O Luderitz* personal communication) (23) —*Rhamno samine* is present in the lipopolysaccharide of *E coli* 03

3 amino 3 6 dideoxy hexoses We have found two representatives of this class of rare amino sugars in the lipopolysaccharides of various *E coli* strains *3 amino 3 6 dideoxy glucose* was found to be a consti tuent of the lipopolysaccharides of O groups 5 65 70 71 and 111 (9 10) This amino sugar had hitherto not been described as a constituent of bacterial lipopolysaccharides However it has been shown recently that this amino sugar is also a constituent of lipopolysaccharides of *Salmonella* (25) *3 amino 3 6 dideoxy galactose* is present in the lipo polysaccharides of *E coli* O groups 2 and 74 (10) It was isolated from

TABLE 2

Cross Reacting Escherichia coli O Antigenic Test Strains

O Group Test Strain			Reaction in Serum against Test Strain of		Sugars Common to Cross Reacting Strains
Chemo type	Strain Number	O Group	O Group	Chemotype	
I	P 6a	32	83	I	BS
			21	II	BS
	H 19a	83	32	I	BS
			21	II	BS
			22	II	BS
II	I 14a	29	76	II	BS GalN
			55	VI	BS GalN
	E 39a	23	39	I	BS
			68	VIII	BS
	F 40	33	19ab	VII	BS
	P 1c	46	57	XX	BS
	F 8d	76	22	II	BS GalN
			87	II	BS GalN
III	G 3404/41	8	46	II	BS
			93	III	BS Man
	H 316	40	75	VIII	BS Man
IV	Bi 7458/41	6	57	XX	BS
V	H 710c	41	87	II	BS
VI	H 35	86	90	VI	BS GalN Fuc
	H 77	90	86	VI	BS GalN Fuc
	Cigleris	128	43	VIII	BS GalN
			87	II	BS GalN
VII	Su 4321/41	13	19ab	VII	BS Rha
			18	VII	BS Rha
			68	VIII	BS Rha
			23	II	BS
			44	XVII	BS
	F 8188/41	19ab	13	VII	BS Rha
			48	VIII	BS Rha
			86	VI	BS
	F 10018/41	18	4	XXI	BS Rha
			25	XXI	BS Rha
			68	VIII	BS Rha
	U 18/41	50	2	XXIX	BS Rha
			74	XXVIII	BS
	Su 3972	54	48	VIII	BS Rha
	F 10167a/41	60	8	III	BS
	H 504c	99	73	III	BS
	178/54	129	13	VII	BS Rha
			26	XXI	BS Rha
VIII	U 8/41	48	37	II	BS GalN
			86	VI	BS GalN
	U 19/41	51	19ab	VII	BS Rha
			21	II	BS GalN
			69	VII	BS Rha
	30w	117	50	VII	BS Rha
XII	Bi 7455	43	36	XXIX	BS Man Fuc

TABLE 2 (contd)

O Group Test Strain			Reaction in Serum against Test Strain of		Sugars Common to Cross Reacting Strains
Chemotype	Strain Number	O Group	O Group	Chemotype	
XIII	H 304	34	85	I	BS
	P 7d	68	1	VII	BS Rha
			25	XXI	BS Rha
	E 49	79	68	XIII	BS Man Rha
			80	VI	BS
XVII	E 10	77	*17*	XIX	GlcN KDO Hep Glc Man
	H 702c	44	73	XVII	BS Man
			68	XIII	BS Man
			77	XVII	GlcN KDO Hep Glc Man
	F 9095/41	59	48	VIII	BS
			57	XX	BS
	P 12a	73	44	XVII	BS Man
			17	XIX	GlcN KDO Hep Glc Man
			68	XIII	BS Man
XIX	K 12a	17	68	XIII	GlcN KDO Hep Glc Man
			77	XVII	GlcN KDO Hep Glc Man
XX	F 7902/41	15	45	XXII	BS FucN
XXI	U 4/41	4	*18*	VII	BS Rha
			68	XIII	BS Rha
	E 47a	25	*68*	XIII	BS Rha
	H 311b	26	68	XIII	BS Rha
XXIV	H 502a	36	68	XIII	BS Man Rha
			141	I	BS
XXVI	P 1a	66	45	XXII	BS 6 de Tal
XXVIII	E 3a	74	*2*	XXIX	BS 3 6-de 3N Gal
			50	VII	BS
XXIX	U 9/41	2	1	VII	BS Rha
			50	VII	BS Rha
			74	XXVIII	BS 3 6 de 3NGal
XXX	U 1/41	5	65	XXX	Bs 3 6 de 3NGlc GalN
			70	XXXI	Bs 3 6 de 3NGlc GalN
			71	XXXII	Bs 3 6 de 3NGlc GalN
	K 11a	65	*70*	XXXI	Bs 3 6 de 3NGlc GalN
			71	XXXII	Bs 3 6 de 3NGlc GalN
XXXI	P 9c	70	*5*	XXX	Bs 3 6 de 3NGlc GalN
			65	XXX	Bs 3 6 de 3NGlc GalN
XXXII	P 10a	71	5	XXX	Bs 3 6 de 3NGlc GalN
XXXIII	26 v	114	5	XXX	BS 3 6 de-3NGlc

a) Titre in heterologous serum [illegible] t more than 4 times lower than that found when serum is tested with its homolog[illegible] train

b) Italic numerals indicate [illegible] l cross reactions

c) Abbreviations used BS = b[illegible] l sugar Glc = glucose Gal = galactose Hep = heptose KDO = 2 keto 3 de[illegible] GlcN = glucosamine Man = mannose GalN = galactosamine Fuc = [illegible] Rha = rhamnose FucN = fucosamine 6 de Tal = 6-deoxytalose 3 6-d[illegible] l = 3 6-dideoxy 3 amino D g[illegible]lactose 3 6-de NGlc = 3 6 d deoxy 3 aminogluc[illegible]e

TABLE 3
Chemotypes of Escherichia coli

Chemotype	3 Amino 3 6 dideoxyglucose	3 Amino 3 6 dideoxy D galactose	2 Amino 2 6 dideoxymannose	2 Amino 2 6 dideoxygalactose	Galactosamine	Glucosamine	2 Keto 3 deoxyoctonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6 Deoxytalose	Colitose	Ribose	Serogroups
I						●	●	●	●	●							14 24 28 30 32 38 42 56 64 8? 83 85 141
II					○	●	●	●	●	●							21 2? 23 27 33 37 46 61 76 81 87
III						●	●	●	●	●	○						8 9 40 58 78 93
IV					○	●	●	●	●	●	○						6
V						●	●	●	●	●		○					41 52
VI					○	●	●	●	●	●		○					80 86 90 127 1?8
VII						●	●	●	●	●			○				1 13 18 19 35 39 50 53 54 60 69 99 100 10? 119 129
VIII					○	●	●	●	●	●			○				48 49 51 117
X						●	●	●	●	●					○		111
XI					○	●	●	●	●	●					○		55
XII					○	●	●	●	●	●	○	○					11 43 1?5
XIII						●	●	●	●	●	○		○				34 68 75 79
XVII						●	●	●		●	○						44 59 73 77
XVIII						●	●	●	●	●	○	○					1?6
XIX						●	●	●		●	○		○				17
XX				○		●	●	●	●	●							1? 15 29 57
XXI				○		●	●	●	●	●			○				4 16 25 26
XXII				○		●	●	●	●	●				○			45
XXIII			○			●	●	●	●	●							3
XXIV						●	●	●	●	●		○	○				36
XXVI						●	●	●	●	●	○			○			66 88
XXVII						●	●	●	●	●		○		○			84
XXVIII		○				●	●	●	●	●							74
XXIX		○				●	●	●	●	●			○				2
XXX	○				○	●	●	●	●	●							5 65
XXXI	○				○	●	●	●	●	●		○					70
XXXII	○				○	●	●	●	●	●			○				71
XXXIII	○					●	●	●	●	●						○	114

= cited from *Kauffmann et al* (17) ● = basal sugars ○ = non basal sugars

Kauffmann et al (17) described *E coli* lipopolysaccharides which do not contain galactose These were classified as chemotypes XVII (O groups 73 and 77) and XIX (O group 17) We have confirmed these findings In the course of our investigations we found two additional galactose less lipopolysaccharides (O groups 44 and 59) However these results were based on paper chromatographic methods Using enzymatic techniques (galactose oxidase) we—and also *O Luderitz* in our laboratories (personal communication) (23) found all lipopoly saccharides mentioned above to contain *small amounts of galactose* (0 6–0 9 per cent) The corresponding glucose values were more than ten times higher (7–12 per cent) For a correct interpretation of these findings further studies are necessary The low galactose content of these lipopolysaccharides can be explained in several ways a) Galac tose is not part of the lipopolysaccharides Galactose containing ma terial is present in the supernatant of the ultracentrifugal sedimenta tion of all galactose less lipopolysaccharides Therefore the small amount of galactose could be constituent of a contamination and not of the lipopolysaccharide b) Galactose is part of the lipopolysacchar ides If so galactose could only be part of the basal core structure of the lipopolysaccharide and not of its specific side chains In such cases the analysis of *E coli* R mutants should be undertaken In suitable R mutants (RII and RI types see (26)) the ratio of galactose glucose should be 1 1 and galactose should occur in larger amounts because of the absence of the long specific side chains provided the core struc ture of *E coli* lipopolysaccharides would be the same as for *Salmonella* Therefore the study of R mutants derived from wild types (S forms) with galactose less lipopolysaccharides appears to be of special interest and is now in progress Until a definite statement can be made strains producing these O antigens are listed as galactose free under chemo types XVII and XIX respectively

Some observations indicate that the basal core structure of *some E coli* lipopolysaccharides may not be the same as that of *Salmonella* When *Luderitz et al* (24) studied partial hydrolysis of the two chemic ally and serologically closely related O antigens of *E coli* O111 and *Salmonella adelaide* (both of chemotype X) they found that specific structures were liberated from the *Salmonella* polysaccharide under laying *Salmonella RI* but following the same procedure from the *E coli* polysaccharide no products with any *Salmonella* RI specificity could be detected The authors therefore concluded that both anti gens although serologically practically identical (identical determi nant groups) differ with respect to their inner core structures of R specificity) Again detailed studies of the lipopolysaccharides of rough mutants (R mutants) derived from *E coli* O test strains would be of great importance for the comparative structural analysis of the basal core of enterobacterial polysaccharides

A comparison of about one hundred *Salmonella* lipopolysaccharides

and about the same number of *Escherichia* lipopolysaccharides appears to be worth while already In both groups the wild types (S forms) produce typical specific *hetero* polysaccharides which are composed of at least 5 different sugar constituents—KDO heptose glucosamine galactose and glucose— the so called basal sugars Most *E coli* lipopolysaccharides contain the same 5 sugars Where galactose appears to be lacking (chemotypes XVII and XIX see above) one could anticipate that mannose replaces galactose but this has to be further elaborated In all chemotypes with the exception of chemotype I (with the 5 basal sugars only) additional sugar constituents are found (see page 344) the most complicated chemotypes representing combinations of 8 different sugars (chemotypes XII XXXI and XXXII) The corresponding lipopolysaccharides are composed of galactosamine the 5 basal sugars and one additional 6 deoxy hexose (fucose or rhamnose) and either mannose or 3 amino 3 6 dideoxy glucose (see Table 3)

Among the known five 3 6 dideoxy hexoses (26) only *colitose* was found in the specific polysaccharides of *E coli* 055 and 0111 All other 3 6 dideoxy hexoses which are characteristic for many *Salmonella* strains (groups A B C D O and Z) have not been found in *E coli* strains

E coli polysaccharides frequently contain various types of *amino hexoses,* such as 2 amino 2 deoxy hexoses 2 amino 2 6 dideoxy and 3 *amino* 3 6 *dideoxy* hexoses thus *giving* rise to several new sugar combinations (chemotypes) 6 deoxy talose has not been found in *Salmonella* but occurs rather widespread in *E coli* polysaccharides Some of the lipopolysaccharides have been investigated earlier by *Kauffmann et al* (17) In most cases there is complete agreement However in the *lipopolysaccharides of E coli 015 025 and 026 fuco*samine was detected in the course of our investigations This has been overlooked by *Kauffmann et al* The lipopolysaccharide of *E coli* 025 was reported (17) to contain galactosamine which we could not verify On the basis of these analytical discrepancies, the chemotype alotment of the lipopolysaccharides has to be changed as follows 015 does not belong to chemotype I but to XX 025 belongs to chemotype XXI (formerly VIII) and 026 to chemotype XXI (formely VII)

The analysis of *E coli* O antigens (lipopolysaccharides) is certainly often complicated by the fact that many *E coli* strains produce a capsular antigen (*K antigen*) and sometimes another mucoid substance (*M antigen*) in addition to the lipopolysaccharide (*O antigen*) These specific polysaccharides of the same strain may have one or several sugar constituents in common but be of quite a different structure (see 38 12) A clearcut decision can be made only after complete separation of these different types of polysaccharides whether or not small amounts of a given sugar are actually part of the very polysaccharide preparation under investigation

Many serological cross reactions between *Salmonella* and *E coli* (and

other enterobacterial) O antigens are known (16) In a comparative study it would be of great interest to estimate the structure of the repeating oligosaccharide units of the specific side chains of such cross reacting lipopolysaccharides

Immunochemical Aspects of E coli *O Antigens*

It is well known that many cross reactions occur among E coli O antigenic test strains (5 13) Some of the established *E coli* test strains are closely related and probably some of the O groups should be brought together under a single label as already proposed by *Ewing et al* a long time ago (5) If such closely related O antigens were to be selected for a more detailed serological analysis the constituting sugars (expressed as chemotypes) would probably serve as a useful guide

TABLE 4
Chemotypes of Escherichia coli *Strains with the Same O Antigen*

Chemo type	Strain number	Serotype			Galactosamine	Glucosamine	2 keto 3 deoxy octonate	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose
		O	K	H									
I	*H 17a*	83	?	31		+	+	+	+	+			
	Buchman	83	?	31		+	+	+	+	+			
I	*RVC 2907*	141	85ab	4		+	+	+	+	+			
	CG 9	141	85ac	–		+	+	+	+	+			
	F 68	141	85ab 88ab	4		+	+	+	+	+			
III	*Bi 316/42*	9	9	12		+	+	+	+	+	+		
	E 69	9	30	12		+	+	+	+	+	+		
III	*G 3404*	8	8	4		+	+	+	+	+	+		
	F 561	8	27	–		+	+	+	+	+	+		
	D 225	8	87 88	19		+	+	+	+	+	+		
	A 295l	8	40	–		+	+	+	+	+	+		
IV	*H 35*	86	–	25	+	+	+	+	+	+		+	
	E 990	86	61	–	+	–	–	+	+	+		+	
	F 1961	86	62	2	+	–	+	+	+	+		+	

The strain numbers of the O antigen test strains are given in italic type

It is a common experience in laboratories where *E coli* determinations are carried out that it is possible to classify up to 90 per cent of newly isolated strains into existing O groups but also that a more detailed antigen analysis of such strains often will lead to the discovery of special (new) antigenic factors Only to a very limited extent have these special O factors been established as official O antigen

SUMMARY

The O antigens of about 100 *Escherichia coli* strains most of them antigenic test strains were isolated in the form of lipopolysaccharides and analysed with respect to their sugar composition 29 different sugar compositions were found and correspondingly 29 chemotypes were established 12 of these were already found to be represented by *Salmonella* strains

In addition to the monosaccharide constituents hitherto found to be common in lipopolysaccharides of *E coli* and *Salmonella* (galactos amine glucosamine heptose galactose glucose fucose rhamnose and colitose) the following sugars were detected fucosamine rhamnos amine 3 amino 3 6 dideoxy glucose 3 amino 3 6 dideoxy D galactose and 6 deoxy talose 2 keto 3 deoxy octonic acid (KDO) was found to be present in all *E coli* strains investigated The number of individual sugars in different lipopolysaccharides ranges from 5 to 8

In the limited number of cases where lipopolysaccharides from dif ferent serotypes with the same O antigen were examined they always were found to have the same sugar constituents (same chemotype) Based on present knowledge about serological relationships between *E coli* O antigens O group cross reactions were compared with the grouping in chemotypes Five out of 14 pairs of cross reacting strains belonged to the same chemotype Furthermore the O antigens of several of the cross reacting pairs grouped in different chemotypes had one or more specific sugar constituents in common especially reciprocal agglutination reactions were found between strains with the same 3 amino 3 6 dideoxy hexose In the limited number of cases where lipopolysaccharides from different serotypes with the same O antigen were examined they were always found to have the same sugar constituents (same chemotype)

The correlation of *E coli* chemotypes to certain biological properties of the respective strains such as their relative pathogenicity and fer tility in conjugation experiments is discussed

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ENTEROTOXIN PRODUCING STAPHYLOCOCCI

A Clinical Bacteriological Study on the Importance of Strains Isolated from Autopsies Wounds and Burns

By

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Received 9 x 67

It is a well known fact that most coagulase positive staphylococci produce a series of enzymes and toxins which since the Bundaberg disaster (13) have been accepted as contributing to their virulence

The importance of the enterotoxins now designated A B and C (4 7) for the symptoms of food poisoning (8 9 11) and staphylococcal enterocolitis (23 32) is well documented However other toxins certainly contribute to the virulence of the staphylococci Special attention has been focused on the different haemolysins

A cytotoxic effect on human cells is exerted by delta lysin and to a minor extent also by alpha lysin (18) Some authors suggest that alpha lysin may induce a shock like state by paralyzing vascular smooth muscle (33 38) Beta lysin has been shown to kill cats and rabbits when injected intra peritoneally or intravenously (13)

The extensive literature on the enterotoxin subject is excellently reviewed by *Elek Kienitz Odenthal* and others (13 22 27) The early symptoms of food poisoning gastroenteritis are due to a direct toxin effect If however bacteria capable of producing enterotoxin multiply in the intestine a severe enterocolitis may develop Severe enterocolitis has also been experimentally induced in the chinchilla with purified preparations of enterotoxins (30 37) The development of staphylococcal enterocolitis is often a nosocomial problem and has been specially noted *after therapy with broadspectrum antibiotics and bowel surgery* Mortality rates of up to 50 per cent have been reported and a toxic course with shock and confusion is seen in connection with food poisoning Enterotoxin producing staphylococci have been relatively difficult to recognize It is therefore not known how common infections with such strains are and to what extent they cause enterocolitis or other types of disease nor is their effect on mortality known

The aim of this report is to elucidate these problems which was made possible by the presence of some closely related multiresistant enterotoxin B producing strains at the University Hospital of Uppsala

in a material from autopsies and plastic surgery and burn cases. The isolated strains were bacteriologically identified by the use of antibiotic sensitivity tests and phage typing. They were also tested with regard to their alpha, beta and delta lysin production, which have been the subject of special interest in this laboratory.

DEFINITIONS

Staphylococcal septic infection or staphylococcal sepsis. The following criteria for staphylococcal septic infection were used.

1. *Autopsy Material*

   a) Growth of coagulase positive staphylococci from autopsy material.
   b) Histopathological signs of infection.
   c) Antemortem symptoms: local clinical signs such as wound sepsis, broncho-pneumonia, enterocolitis, leucocytosis and/or general clinical symptoms such as fever and prostration.

However, in three cases of ulcerating tumours of the lung, clinical and bacteriological evidence alone was accepted as criteria. Wound sepsis was not microscopically verified.

2. *Clinical Material from Plastic Surgery and Burn Cases*

Growth of coagulase positive staphylococci from suppurating wounds was accepted as proof of septic infection.

Mixed septic infection with Staphylococcus aureus as primary organism. This term means that besides staphylococci other bacteria were isolated from autopsy material. The staphylococci, however, were considered to be of dominant importance if they had been isolated as the primary organism from blood or a septic site during life.

MATERIALS AND METHODS

Clinical Materials

Septic infections with coagulase positive staphylococci were studied in necropsies and in patients treated at a plastic surgery and burns unit. In the first group all infections with coagulase positive staphylococci were studied in detail. In the second group only cases infected with enterotoxin B producing staphylococci were studied to the same extent.

1. *Autopsy Material*

Between January 1st, 1964 and April 30th, 1965, 256 autopsies were examined bacteriologically. The selection of these cases was made by the pathologists. Coagulase positive staphylococci were isolated from 58 cases. Twelve cases not fulfilling the criteria of septic infection were excluded.

The remaining 46 patients comprise a heterogeneous group with respect to age (3 days–89 years), type and duration of disease. The distribution in age groups is shown in Fig. 1. Twenty three of the patients were 60 years or older. There was a 3 : 2 ratio in favour of males. Many of the patients had suffered from a severe underlying disease which is illustrated in Table 1. Two of the patients were registered for two diseases.

Thirty eight of the patients came from wards in the University Hospital of Uppsala, three cases were admitted to the Ulleråker Hospital for mental diseases, two cases to the Samariter Hospital and three to the Hospital for Infectious diseases. The infections were relatively evenly spread over the period, at no time reaching epidemic proportions.

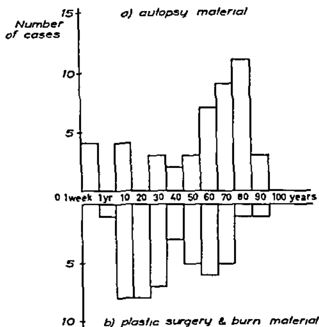


Fig 1

Distribution in age groups of different clinical materials

TABLE 1

Associated Pre Existing Diseases in 46 Cases of Staphylococcal Sepsis Studied by Post Mortem Examinations

Disease	Cases
Congenital diseases or premature infants	5
Malignancy without operation	12
Malignancy with operation	4
Sarcoidosis	1
Bronchial asthma	1
Aplastic anaemia or agranulocytosis	2
Diabetes mellitus	2
Cirrhosis hepatis	1
Pyelonephritis	3
Pyoderma gangrenosum	1
Abortion	1
Third degree burn	6
Fracture	
Gastric ulcer with gastri rese tion	1
No clear underlying di ea e	6

b Plastic Surgery and Burn Mater al

This material was collected durin t o per ods in 1966 betw en April 2?nd–July 22nd and July 23rd–October 31st and t t d me what different as will be described in detail under "Sampling" All p t t w e treated in two wards in the Depart ment of Plastic Surgery Univer it H spital Uppsala Ei hty eight cases with staphylococcal wound sepsis were reg tered—43 during the first and 45 during the second period

Forty five of these (23 and 22 w r at some time infected with enterotoxin B producing staphylococci Distribut n n a e groups and types for pre existing dis eases are shown in Fig 1 and Table

TABLE 2
Associated Pre Existing Conditions in 45 Cases of Wound Sepsis

Burns	25
Cold injury	1
Traumatic wounds	7
Malignancy	4
Chronic ulcerations	3
Skin grafts	5

Sampling

1 *Autopsy Material*

Specimens for bacteriological examination were taken from lung heart blood kidney spleen and other organs when indicated Specimens were taken from the organs with sterile instruments according to *Adamsson* (1) The surface of the *removed tissue* was flamed and brought to bacteriological examination in sterile Petri dishes This technique has recently been reevaluated by *Olding* (28) All isolated coagulase positive staphylococci were kept in stock as freeze dried broth cultures

2 *Plastic Surgery and Burn Material*

To find out whether a strain had been acquired in the hospital nose and throat *specimens were taken from all patients on admission*

At the start of the first period specimens were from all patients from nose throat faeces and if present from pus Faecal specimens were taken with rectal swabs *The specimens were investigated with regard to growth of enterotoxin B* positive staphylococci and other bacteria Following this first investigation specimens were routinely taken from suppurating wounds usually once a week. When *enterotoxin positive staphylococci were isolated specimens were also taken from* nose throat and faeces In 7 out of 23 cases no specimens from nose throat or faeces were obtained

During the second period no specimens were collected from nose throat or faeces but from suppurating wounds as before

All enterotoxin B producing staphylococci were kept freeze dried

Clinical Study

1 *Autopsy Material*

The clinical records were carefully reviewed in conjunction with the autopsy records and all signs of local or general infection noted

2 *Plastic Surgery and Burn material*

Patients with enterotoxin B positive strains in wounds were studied with regard to *mortality symptoms of general infection such as fever shock diarrhoea and con*fusion evidence of local wound sepsis such as delayed healing

Antibiotic Policy

Antimicrobial drugs were given to 33 among the 45 patients comprised in the plastic *surgery and burn material Penicillins alone—most benzylpenicillin and ampicillin* —were given to 25 patients Another 4 patients received penicillins but for some time also broad spectrum antibiotics Four patients received only broad spectrum *antibiotics Because of its great heterogeneity* the autopsy material was not consequently studied with regard to antibiotic policy

Bacteriological Technique

Culture and Media

a) All wound and autopsy specimens were inoculated on meat infusion agar with 10 per cent sheep s blood gentian violet agar chocolate agar Chapman agar and ordinary broth for enrichement Anaerobic cultur [illegible] ere undertaken on sheep blood agar and thioglycollate medium

b) Specimens from nose throat and rectum were cultivated on blood agar (aerobically) Chapman agar and nutrient broth containing 6 5 per cent sodium chloride for enrichement

For toxin production the strains were cultivated on a semisynthetic solid medium modified according to *Casman* and covered with sterile cello phane sheath as de scribed earlier (16)

En yme and Toxin Assay

a) Test for coagulase was performed on slides and in tubes according to *Duthie* (1?)

b) Enterotoxins A and B were tested in gel diffusion against specific antisera according to *Wadsworth* (35) In some large scale experiments the modification with LKB standard equipment (17) was used One per cent agarose (20) in barbital buffer pH 8 6 (LKB 3?76–AB) was used as supporting medium in both tests

Antienterotoxin A serum was kindly supplied by Dr *E Casman* Antienterotoxin B serum was produced as described in a previous report (17) The former was used undiluted the latter in a dilution of 1/10 Normal antibodies present in pre immunesera were absorbed with boiled Wood–46 bacteria Strains producing entero toxin A only and some selected strains producing neither enterotoxin A nor B but isolated from cases with enterocolitis were in addition examined by cat test accord ing to *Hammon* (19) with the modification that haemolysins were neutralized with staphylococcal antitoxin (Burroughs Wellcome et Co lot nr RA 388 ?0000 units in 29 ml) before injection It was controlled in order to exclude a presence of alpha beta and delta lysin activity

The autopsy strains were tested for enterotoxin twice immediately after isolation and again after they had been lyophilized in association with tests for haemolysins

c) Tube serial dilution tests for alpha beta and delta lysins were performed as described in previous papers (15 18) The results were read as haemolysis after 1 hour at 37 C and 1 hour at 4 C All strains were grown on the same batch of medium and tested with the same batch of red cells from rabbit, sheep and man respectively

Antibiotic Sensitivity Test

The disc diffusion method according to *Ericsson et al* (14) was applied for anti biotic sensitivity tests Ordinary sheep blood agar plates were used

Phage Typing

The technique described by *Blair & Williams* (1961) (6) was used. For the autopsy strains the 21 international basic" phages described in their paper were utilized together with D 84 85 (21) KS6 (3?) 86 (34) and 83A (31) The slightly revised phage set recommended at the Moscow meeting July ? nd 196? (31) was used for the plastic surgery and burn material The phages 83A 84 and 8 were used only in RTD All phage typing was performed with reference strains

Histopathological Diagnosis

All autopsies were performed by the staff of the Department of Pathology Univer sity of Uppsala and their records were used with kind permission by the prefect

RESULTS

Analysis of Bacteria

Isolation of Enterotoxin Producing Staphylococci and other Organisms

1) *Autopsy material* Table 3 shows the total spectrum of isolated bacteria Coagulase positive staphylococci were isolated from three or more organs in 24 of the 46 cases In 8 of these staphylococci only were isolated In addition to the aureus staphylococci which constitu

ted the basis for selection other organisms were also encountered These were mainly coliforms and other members of the normal intestinal flora

Altogether staphylococci were present as the only organism in 13 cases and as the primary organism in a further 7 cases

When the coagulase positive staphylococci were phage typed double *infections were demonstrated in 4 cases*

TABLE 3

Staphylococcus aureus *and Other Microorganisms Isolated at Autopsy from 46 Cases*

Isolated organisms	3 organs or more		1–2 organs	
	more than one species	staphylococci only	more than one species	staphylococci only
Staph aureus	16	8	17	5
Staph albus			3	
Alpha strept			1	
Enterococci	3		4	
Coliforms	11		15	
Proteus	6		2	
Pyocyaneus	1		1	
Fungi	2			

TABLE 4

Frequency of Enterotoxin Production in 50 Strains

	Number of cases
Enterotoxin type A	2
Enterotoxin type B	18
Enterotoxin type ?	1
Total in 50 strains	21 (42%)

As demonstrated in Table 4 21 of the 50 isolated staphylococcal strains produced *some type of enterotoxin* Eighteen were of type B 2 of type A one strain being of unknown antigenic type None of them produced both enterotoxin A and B Only eleven of the strains which produced enterotoxin B did so in titres as high as 1/20 to 1/160 and two of them lost their toxinproducing capacity after freeze drying

Thirteen of the enterotoxin producing strains belonged to the group of only or primarily recovered organism

2) *Material collected at the Department of Plastic Surgery* Enterotoxin B positive staphylococci which constituted the basis for selection in this material were isolated *from 45 patients with suppurating* wounds

Specimens from nose throat and faeces were taken from 17 of these patients during the first period In 13 the strain of the wound was also isolated from faeces and in 16 cases from nose or throat

In addition to the staphylococci other bacteria were on various occasions cultivated from 31 out of the 45 patients (approx 70 per cent) Beta haemolytic streptococci were found in 16 cases pyo cyaneus bacteria in 6 proteus bacteria in 6 and coliforms in 10 cases Enterococci alpha streptococci and fungi were found sporadically

Phage Typing and Antibiograms

1) In the autopsy material 12 of the coagulase positive staphylo cocci belonged in phage group I—(Table 5)—five in group II and 12 in group III Phage groups I and III were represented by strains with various patterns All 5 strains in group II were lysed by the phage 3A (1000×RTD) only Twenty of the strains were non typable The phagetypes of enterotoxin positive strains are presented in Table 6 Only 6 of 21 strains were typable 4 of them however only in 1000× RTD The UC 18 phage lysed three strains

TABLE 5

Coagulase Positive Staphylococci Isolated at Autopsy Distributed in Phage Groups

I	12
II	5
III	12
I + III	1
NT	20
Total	50

TABLE 6

Phage Types of Enterotoxinproducing Strains Isolated at Autopsy

Phage types	A	B	Other
84§		1	
47/86		1	
83A/86 + 84§		1	
4 E/86	1		
80/KS6/42E/47/83A + D	1		
77/84§		1	
NT		14	1

1000 × RTD § RTD

All of the 14 B strains distinguished as non typable gave irregular inhibition patterns with group III phages and variable reactions with phage 29

The antibiograms in Fig 2 demonstrate however that the 14 non typable strains were not quite identical from that point of view Multi resistance characterized all of them but some were sensitive to chlo

ted the basis for selection other organisms were also encountered These were mainly coliforms and other members of the normal intestinal flora

Altogether staphylococci were present as the only organism in 13 cases and as the primary organism in a further 7 cases

When the coagulase positive staphylococci were phage typed double infections were demonstrated in 4 cases

TABLE 3

Staphylococcus aureus and Other Microorganisms Isolated at Autopsy from 46 Cases

Isolated organisms	3 organs or more		1–2 organs	
	more than one species	staphylococci only	more than one species	staphylococci only
Staph aureus	16	8	17	5
Staph albus			3	
Alpha strept			1	
Enterococci	3		4	
Coliforms	11		15	
Proteus	6		2	
Pyocyaneus	1		1	
Fungi	2			

TABLE 4

Frequency of Enterotoxin Production in 50 Strains

	Number of cases
Enterotoxin type A	2
Enterotoxin type B	18
Enterotoxin type ?	1
Total in 50 strains	21 (42%)

As demonstrated in Table 4 21 of the 50 isolated staphylococcal strains produced some type of enterotoxin Eighteen were of type B 2 of type A one strain being of unknown antigenic type None of them produced both enterotoxin A and B Only eleven of the strains which produced enterotoxin B did so in titres as high as 1/20 to 1/160 and two of them lost their toxinproducing capacity after freeze-drying

Thirteen of the enterotoxin producing strains belonged to the group of "only or primarily recovered organism

2) *Material collected at the Department of Plastic Surgery* Enterotoxin B positive staphylococci which constituted the basis for selection in this material were isolated from 45 patients with suppurating wounds

Specimens from nose throat and faeces were taken from 17 of these patients during the first period In 13 the strain of the wound was also isolated from faeces and in 16 cases from nose or throat

In addition to the staphylococci other bacteria were on various occasions cultivated from 31 out of the 45 patients (approx 70 per cent) Beta haemolytic streptococci were found in 16 cases pyocyaneus bacteria in 6 proteus bacteria in 6 and coliforms in 10 cases Enterococci alpha streptococci and fungi were found sporadically

Phage Typing and Antibiograms

1) In the autopsy material 12 of the coagulase positive staphylococci belonged in phage group I—(Table 5)—five in group II and 12 in group III Phage groups I and III were represented by strains with various patterns All 5 strains in group II were lysed by the phage 3A (1000×RTD) only Twenty of the strains were non typable The phagetypes of enterotoxin positive strains are presented in Table 6 Only 6 of 21 strains were typable 4 of them however only in 1000× RTD The UC 18 phage lysed three strains

TABLE 5

Coagulase Positive Staphylococci Isolated at Autopsy Distributed in Phage Groups

I	12
II	5
III	12
I + III	1
NT	20
Total	50

TABLE 6

Phage Types of Enterotoxinproducing Strains Isolated at Autopsy

Phage types	A	B	Other
84§		1	
47/86		1	
83A/86 + 84§		1	
42E/86	1		
80/K56/42F/47/83A + D§	1		
77/84§		1	
NT		14	1

1000 × RTD § RTD

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bit sheep and human erythrocytes gives an approximate understanding of the production of alpha beta and delta haemolysin respectively Each haemolysin was not produced (or was produced in a very low titre) by approximately 20 per cent of the strains Most of the strains produced the toxins in moderate titres (1/8–1/80) A strong haemolytic effect on rabbit erythrocytes— Alpha lysin —was given by 15 strains Ten of these also produced enterotoxin—all of type B— Among eleven strains which produced toxin that haemolysed sheep erythrocytes in high titres (> 1/640) with the hot cold phenomenon typical for beta lysin four also produced enterotoxin B These four strains were all non typable

2) Most of the strains isolated from suppurating wounds and burns also produced haemolysins in moderate to high titres Delta haemolysin was as a rule produced in high titres only

TABLE 7

Haemolytic Capacity of 48 Autopsy Strains 19 of Which Were Enterotoxinproducing and 52 Wound Strains

	Number of strains	*Haemolytic titre all autopsy strains* <1/8	1/8–1/80	1/160–1/640	>1/640
rabbit erythrocytes	48	10	23	15	0
sheep	48	10	24	3	11
human	48	9	34	5	0
		Haemolytic titre enterotoxinproducing autopsy strains			
rabbit erythrocytes	19	5	4	10	
sheep	19	5	9	1	4
human „	19	6	9	4	
		Haemolytic titre wound strains			
rabbit erythrocytes	52	2	29	21	
sheep	52	1	34	6	11
human	52	2	5	43	2

Clinical Syndromes

1) *Autopsy Material*

Table 8 shows that enterocolitis —a diagnosis based on clinical as well as histopathological observations—was found in seven cases Enterotoxin B producing strains occurred in three enterotoxin A producing strains in two and staphylococci producing enterotoxin of unknown antigenic type in one of these cases One case of enterocolitis was also found in the enterotoxin negative group a one year old mongoloid child

In 39 cases of staphylococcal septic infection without symptoms of

enterocolitis enterotoxin B producing staphylococci were isolated from 15 and enterotoxin negative strains from 24 cases No staphylococci producing enterotoxin A or enterotoxin of unknown antigenic type were isolated from this group Most patients infected with enterotoxin positive strains in this group suffered from pneumonias and/or wound sepsis

TABLE 8

Different Types of Local Infection Patterns in 46 Autopsy Cases

Type of infection	Number of cases	Enterotoxin positive 21 cases			Enterotoxin negative 25 cases
		Enterotoxin Type A (2)	Enterotoxin Type B (18)	Enterotoxin Type ? (1)	
Infections with enterocolitis as one symptom					
Enterocolitis	2	1	–	1	–
Enterocolitis + *infected wound*	4	1	2	–	1
Enterocolitis + *infected burn*	1	–	1	–	–
	7	2	3	1	1
Pyogenic infections without enterocolitis					
Respiratory	28		11		17
Infected burn	5		4		1
Abscess	11		2		9
Septic abortion	1		1		–
Peritonitis	1		–		1
Meningitis	1		–		1
Total	47		18		29

Bacteria could at autopsy be isolated from blood culture and/or parenchymatous organs in 38 cases In 18 of these enterotoxin B producing staphylococci were isolated in one enterotoxin A producing and in 19 enterotoxin negative strains

As many of the patients had more or less severe underlying diseases and some of them were infected with other bacteria the importance of the staphylococci was evaluated in each case Some case records of special interest will be described in more detail

Case Descriptions (Autopsy material from various wards)

1 A F a thirty seven year old woman treated since 1961 for an indefinite neuro logical disorder tics being the predominant symptom On February 12th 1964 she was operated on for a perforated gastric ulcer *Five days later* the wound in her abdominal wall ruptured and she developed profuse diarrhoea Coagulase positive staphylococci were isolated from the faeces *The diarrhoea could not be controlled* and the patient died on April 22nd 1964 *Enterotoxin A producing staphylococci* NT

1000 × RTD 42F/86 III—were isolated from her heart blood lung liver kidney spleen and intestine The strain also produced alpha lysin 1/160 but beta and delta lysin less than 1/8 In the autopsy record the cause of death was recorded as staphylococcal enterocolitis of pseudomembraneous type + septicaemia + bleeding into the left nucleus caudatus of the brain

2 S A a fifty two year old woman became ill on the 10th September 1964 with profuse diarrhoea and pyrexia up to 41 C. She died on the 15th Sept 1964 Coli bacteria were isolated from her heart blood lung spleen kidney and intestine From the intestine were also isolated enterotoxin A producing staphylococci NT 1000 × RTD 80/KS6/42 F/47/83 A The isolated strain also produced alpha lysin 1/80 and beta and delta lysin 1/16 and 1/32 respectively Cause of death was given as agranulocytosis? + aplastic anemia + pseudomembraneous colitis + septicaemia

3 C.L. a four year old girl was scalded by boiling water on the 26th September 1964 and received a 20 per cent scald of 2nd degree She was hospitalized and prophylactically treated with penicillin Diarrhoea started on the 30th of September Staphylococci NT producing enterotoxin B and alpha and delta lysin in titres 1/320 and 1/40 respectively were isolated from her burns faeces nose and throat This strain was not present in her nose or throat on admission on Oct 2nd On the day of her death staphylococci of the same phage type were isolated from her blood together with staphylococci of phagetype 80/81 I This strain produced alpha and delta lysin in titres 1/40 and 1/10 respectively but no enterotoxin or beta lysin These two strains were also isolated at autopsy Cause of death was given as scalds + Septicaemia Macroscopically and histologically there were very insignificant signs of enteritis

4 K Ö a sixty seven year old woman sustained a third degree burn of 20 per cent on April 1st 1964 The burn was excised on April 16th and 21st and the wound surface grafted on April 23rd However after the last operation she developed serious wound sepsis and shock complicated by frequent episodes of diarrhoea during her last week of life She died on May 7th 1964 The same coagulase positive staphylococci NT 1000 × RTD 47/86 III which were isolated from wound blood and faeces *in vivo* were at autopsy isolated from her heart blood lung kidney spleen and liver together with *E coli* The staphylococci produced enterotoxin B but no alpha beta or delta lysin Cause of death was given as burns + Septicaemia Macroscopically there were virtually no signs of enteritis

5 E.L. a seventy two year old man with carcinoma of the rectum was operated upon on April 12th 1963 and rectal amputation and colostomy performed On the 17th April he developed a severe enteritis ending in anuria and shock He died on April 20th At autopsy staphylococci NT together with *E coli* and enterococci were isolated from his blood lung kidney spleen liver and intestine The strain produced enterotoxin B alpha lysin in titre 1/320 beta lysin in titre 1/5120 and delta lysin in titre 1/160 The enterocolitis was of the pseudomembraneous type

6 M B A., a thirty three year old woman with a septic abortion was brought to an obstetric ward on March 12th 1964 because of hyperemesis She became acutely confused and was transferred via a mental hospital to an intensive care ward where she died in a state of septic shock 3 days later on March 18 Earlier she had never shown any mental disorder At autopsy enterotoxin B positive staphylococci NT producing also alpha beta and delta lysin in haemolytic titres of 1/320 1/40 and 1/20 respectively were isolated from her heart blood lung spleen kidney and uterus Histopathology showed small septic haemorrhages and signs of early tubular nephritis

7 F L. a forty eight year old previously healthy man was admitted on the 10th February 1964 to the Ulleråker mental hospital as a case of acute catatonic crisis" On February 23rd he was transferred to an intensive care unit with high fever He died on March 6th with extensive pneumonia lungs shock and oliguria At autopsy staphylococci NT together with *E coli* were isolated from blood lung kidney and spleen The staphylococci produced enterotoxin B As this capacity was lost after freeze drying the strain was not further investigated with regard to haemolysins

8 E.D a seventy five year old woman—together with 2 other ladies in a home for the elderly—started vomiting on September 3rd 1964 and developed diarrhoea The patient was admitted to the University Hospital on September 28th The other two soon recovered Coagulase positive staphylococci NT 1000 × RTD NT were isolated from her faeces on five different occasions The strain produced neither

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Considering the 15 cases in which staphylococci only were isolated fever probably of infectious origin occurred in 3 cases and in 6 wound healing was delayed It was observed that staphylococci of this type isolated as the only organisms necrotized and damaged skin grafts in a similar way to beta haemolytic streptococci The evaluation of the influence of staphylococcal infection on fever and wound healing was more difficult when double infections were present usually caused by beta haemolytic streptococci and gram negative rods In 15 out of 18 cases with fever suggesting a probable infection and in 12 out of 18 cases with delayed wound healing other pathogens were isolated at the same time

The co existance of these staphylococci and beta haemolytic strepto cocci constituted a special therapeutic problem which required the application of broad spectrum antibiotic ointments

Epidemiological Observations

A complete analysis of the epidemiological situation was not the object of this work Still some data of importance will be noticed

A group of closely related strains—non typable multiresistant and enterotoxin B producing—has been present at the University Hospital of Uppsala for at least three years They have been isolated in many different wards

Fourteen patients comprised in the autopsy material were infected with such strains and it is interesting to note that 9 of these at least for some time had been treated in the intensive care ward where the same staphylococci were isolated from healthy staff members as well as from a staff member with a minor wound sepsis These bacteria were also found in the environment in the intensive care ward In addition to the patients harbouring these strains the autopsy material represented a very heterogeneous group of patients from several differ ent wards The isolated staphylococci belonged to several various phagetypes

The material collected in the Department of Plastic surgery was of a more homogeneous nature Approximately 50 per cent of the patients with staphylococcal wound sepsis were infected with variants of these closely related bacteria There is evidence that 39 out of 46 patients comprised in the material were infected after admission to the depart ment Six patients probably brought their infections along from other hospitals The contribution from other sources is further reflected in the fact that among the 3 strains typable by bacteriophages in RTD two—84/85 III and 85 III—belong d in this latter group

DISCUSSION AND CONCLUSIONS

Some earlier papers from this laboratory have dealt with the problem of producing large amounts of enterotoxin in staphylococcal cultures

and the production of specific antienterotoxin B sera (16 17) A method was developed which simplifies the investigation of a large number of strains for enterotoxin production It was used in this study on strains isolated at necropsies and from suppurating wounds and burns

Among 50 coagulase positive staphylococci isolated at post mortem examinations of 46 cases with clinical and microscopic evidence of septic infection 21, or approx 40 per cent were found to produce enterotoxin of some type 18 type B 2 type A and one of unknown type No serum was available to determine whether the latter was of the newly described type C (4) It is surprising to find such a high frequency of enterotoxin producers In thirteen cases they were considered to be of clinical importance which establishes the virulence involved but it also raises the question whether enterotoxin enhances virulence The accumulation of enterotoxin positive strains however can not be taken as proof of such a theory The heterogeneity and the existence of predisposing diseases make autopsy materials less suitable for the evaluation of virulence a problem met with also by other workers (25 29)

A detailed study of the enterotoxin positive strains revealed that *14 B strains were closely related They were all multiresistant with* variations in sensitivity to erythromycin and chloramphenicol The strains were shown by *Barber s* method (3) to have increased resistance to methicillin though varying results were found with the disc diffusion method (to be published separately) In phage typing they were non typable but in 1000 × RTD they often gave reactions with phage 29 group I and irregular inhibition patterns with group III phages This is rather unusual since most enterotoxin producers are lysed by group III phages only (13 22 27) The UC 18 phage suggested by *Altemeier et al* (2) as being specific for enterotoxin producing staphylococci lysed only three of 21 strains in this material

At closer investigation these strains were isolated in many wards but mainly the Department for Plastic Surgery The antibiograms and the capacity to produce enterotoxin B served as the most useful means of identification During six months they were isolated from 45 patients with suppurating wounds and burns often in association with other bacteria This material was used to evaluate the risks of infection with enterotoxin producing staphylococci and can be regarded as a control of the autopsy material No attempt was made to study non enterotoxigenic strains Most septic infections were mild There were only two fatal cases This might be because the patients were mostly healthy and well nourished Debilitated patients are more prone to develop severe infections and this was reflected in the autopsy material However a delay in wound healing a bad take and necrosis of the skin grafts were noticed in some patients who did not have mixed infection In other papers (24) staphylococci in burns have

been considered of minor clinical importance Thus these results do not lend much support to the theory that enterotoxinproducing strains are more virulent The accumulation of enterotoxin producing stap hylococci in the autopsy material might well be due to the epidemic situation in the wards concerned

Clinically enterotoxin has been considered responsible for entero colitis and food poisoning Seven cases of severe enterocolitis were observed in the autopsy material It is interesting to note that only three of these were infected with bacteria producing enterotoxin B which was earlier called the E or enteric type (7) In two cases staphylococci producing enterotoxin A (the F or food poisoning type) were isolated and in one staphylococci producing enterotoxin of unknown antigenic type Five cases were of pseudomembraneous type (13 22 27) while two B infected cases only showed slight micro scopic evidence of enterocolitis In most cases described in the litera ture the symptoms had developed after therapy with broad spectrum antibiotics (13 22 27) In this material only one of the cases had been treated with oxytetracyclin before onset of symptoms In two patients symptoms developed after gastric or bowel surgery Throughout the period concerned the plastic surgery and burn material comprised only three cases of mild diarrhoea in which the outcome was not fatal although enterotoxin producing strains were isolated from rectal swabs from 13 out of 17 patients All but three strains of enterotoxin B producing staphylococci were thus isolated from patients in whom signs of enterocolitis were absent Similar experiences have been re ported by *Lavine et al* (26) who however only tested 10 type strains for enterotoxin production The remaining strains were tested indi rectly by phage typing

In the autopsy material most strains were isolated from cases of bronchopneumonia and from infected wounds In the clinical material suppurating wounds represented the criterion for selection

The final role of enterotoxin remains obscure since many of the enterotoxin producing strains also produced haemolysins in high titres The haemolytic capacity was given only as the effect of crude un neutralized toxin on rabbit sheep and human erythrocytes indicating the presence of alpha beta and delta haemolysin In any case the haemolysins act together *in vi* The titres present approximative comparison values because there is a difference in haemolytic activity between various colonies of the same strain (5)

It is evident that strains producing enterotoxin only rarely were responsible for a development of enterocolitis but more often appeared in other types of staphylococcal infections These variations may be explained by differences in the patients susceptibility Why these strains were so widely spread in the hospital remains to be defined

SUMMARY

In an autopsy series comprising 46 cases of infection with coagulase positive staphylococci 18 out of 50 isolated strains produced enterotoxin B two enterotoxin A and one enterotoxin of unknown antigenic type Strains which produced high titres of enterotoxin B frequently also produced high titres of alpha- and delta haemolysin Neither alpha beta or delta haemolysin was produced by all strains Fifty two enterotoxin B producing strains isolated from suppurating wounds and burns produced moderate to high titres of alpha beta and delta haemolysin

The importance of enterotoxins in the development of enterocolitis is substantiated by the fact that at least 6 out of 7 cases of severe enterocolitis were infected with enterotoxin producing strains Three of these produced enterotoxin type B two type A one producing the unknown type However enterotoxin B strains were also isolated from many patients with other types of septic infection and from patients in whom signs of enterocolitis were absent

This observation was further *emphasized by the results from a* plastic surgery and burn material Enterotoxin B producing strains were isolated from suppurating wounds in 45 cases Two patients died in a state of septic shock and 3 other patients developed mild diarrhoea In 13 out of 17 investigated cases the strain was also harboured in the stools Necrosis of skin grafts was observed in some cases

Most enterotoxin B producing strains in both series represented variations of a multiresistant non typable strain endemic in some wards of the University Hospital

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SUMMARY

In an autopsy series comprising 16 cases of infection with coagulase positive staphylococci, 18 out of 50 isolated strains produced enterotoxin B, two enterotoxin A and one enterotoxin of unknown antigenic type. Strains which produced high titres of enterotoxin B frequently also produced high titres of alpha and delta haemolysin. Neither alpha, beta or delta haemolysin was produced by all strains. Fifty two enterotoxin B producing strains isolated from suppurating wounds and burns produced moderate to high titres of alpha, beta and delta haemolysin.

The importance of enterotoxins in the development of enterocolitis is substantiated by the fact that at least 6 out of 7 cases of severe enterocolitis were infected with enterotoxin producing strains. Three of these produced enterotoxin type B, two type A, one producing the unknown type. However, enterotoxin B strains were also isolated from many patients with other types of septic infection and from patients in whom signs of enterocolitis were absent.

This observation was further emphasized by the results from a plastic surgery and burn material. Enterotoxin B producing strains were isolated from suppurating wounds in 1[illegible] cases. Two patients died in a state of septic shock and [illegible] other patients developed mild diarrhoea. In 1[illegible] out of 17 investigated cases the strain was also harboured in the stools. Necrosis of skin grafts was observed in some cases.

Most enterotoxin B producing strains in both series represent [illegible] variants of a multiresistant non typable strain endemic in some wards of the University Hospital.

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THE IMMUNOLOGICAL SPECIFICITY OF FLUORESCENT ANTI *YERSINIA ENTEROCOLITICA* GLOBULIN

By

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Received 17 v 67

Yersinia enterocolitica (syn *Pasteurella X*) has recently attracted attention as a probably common cause of certain abdominal affections The organism had formerly been isolated from man in only a few cases (*Hassig et al* 1949 *Carlsson et al* 1964 *Mollaret & Chevalier* 1964 *Wauters & Mollaret* 1965 *Graux & Wauters* 1966) but in 1966 *Winblad et al* found it to be common in the intestinal flora with corresponding antibody production in patients with symptoms suggesting appendicitis and particularly in acute terminal ileitis and mesenteric lymphadenitis Later investigations lent further support to the assumption of the above mentioned aetiological role of this organism (*Winblad Nilehn & Jonsson* 1966 *Nilêhn & Sjostrom* to be published)

In recent years the fluorescent antibody (FA) method has been used more widely for the demonstration of bacteria in clinical specimens Thus the method has been successfully used with a view to demonstrating inter alia enteropathogenic *E coli* (*Whitaker et al* 1958 *Danielsson & Laurell* 1961 gonococci (*Deacon et al* 1959 *Danielsson* 1963) and group A streptococci (*Moody et al* 1963)

Isolation of *Yersina enterocolitica* from culture of faeces is time consuming and requires considerable experience on the part of the examiner A simpler and quicker method fulfilling at least requirements of a screening test is desirable It was therefore thought worth while to try the FA method according to the direct technique in which smears of faecal samples are stained by fluorescent anti *Y enterocolitica* globulin

In the present work the immunological spectrum of fluorescein isothiocyanate labbelled anti *Y enterocolitica* rabbit globulin was studied

MATERIAL AND METHODS

Y enterocolitica strains Out of 20 strains examined 12 were isolated from man (336 (*Hassig* 1959) Winblad (*Carlsson et al* 1964) 6493 5674 5653 5788 6064 5944

The investigation was supported by grants from the *Medical Faculty of the University of Lund* and the *Alfred Österlund Foundation* Malmo

144, 45[illegible]8, 5525, 564 (*Vilén* 1967)) and 8 from chinchilla (1975, 1905, 924, 931 (*Daniels* 1963), [illegible]63 (*Siegmann* 1963), 18, 51 (*Becht* 1962), P 76 (*Frederiksen* 1964)).

For immunization of rabbits and for further immunological studies, use was made of a human strain (strain "Winblad") and three chinchilla strains) (*Frederiksen* P 76, *Becht* 51 and *Daniels* 924). The latter were selected with reference to *Winblad's* investigations of type specific O antigen factors of *Y. enterocolitica* (to be published). The four strains behaved like different antigenic types and are hereinafter designated human strain ("Winblad"), ch I (Frederiksen P 76), ch II (Becht 51) and ch III (Daniels 9[illegible]4).

The strains were cultured on blood agar plates and used for the preparation of smears on microscopic slides after incubation for 24 hours at 22 °C.

Heterologous bacterial strains. The conjugatees were tested with [illegible]58 non *Y. enterocolitica* strains (Table 1). Most of them had been isolated from routine samples from patients in Malmö General Hospital. Each strain was cultured on the medium most suitable for its growth requirements.

TABLE 1

The Reactive Properties of FITC Labelled Anti Yersinia enterocolitica *Globulin and Normal Rabbit Globulin with Different Bacterial Strains*

Bacterial strains tested	Number of strains	FITC-labelled anti Y. enterocolitica (human strain) rabbit globulin		FITC labelled normal rabbit globulin
		1:40	1:160	1:40
Yersinia enterocolitica, human strains	12	4+	3–4+	0
Yersinia enterocolitica, chinchilla strains	7	4+	3–4+	0
Yersinia enterocolitica, chinchilla strain	1	0	0	0
Yersinia pestis	1	0–1+	0	0
Yersinia enterocolitica, type I, II, III, IV, V	5	0	0	0
Pasteurella multocida	14	0	0	0
Escherichia coli, unspec. types	20	0	0	0
Escherichia coli O 128 B 12	1	0	0	0
Escherichia coli O 86 B 7	1	0	0	0
Hafnia spp	5	0	0	0
Unspecified lactosefermenting coliforms	40	0	0	0
Klebsiella Aerobacter spp	10	0	0	0
Proteus mirabilis, vulgaris, rettgeri, morganii	21	0	0	0
Pseudomonas pyocyanea	11	0	0	0
Bacterium anitratum	4	0	0	0
Salmonella types from groups B, C, D, E	17	0	0	0
Shigella sonne	2	0	0	0
Bacteroides spp	5	0	0	0
Neisseria gonorrhoeae	[illegible]	0	0	0
Neisseria catarrhalis, sicca, flava, flavescens	18	0	0	0
Veillonella spp (salivary strains)	11	0	0	0
Veillonella like faecal strains	3	4+	3–4+	0
Staphylococcus aureus	9	0	0	0
Staphylococcus aureus	[illegible]	4+	3–4+	4+
Staphylococcus albus	10	0	0	0
Streptococcus from groups A, C, G	5	0–1+	0	0–1+
Viridansstreptococci	6	0	0	0
Enterococci	24	0	0	0
Pneumococcus	2	0	0	0
Listeria type I, II, IVb	3	0	0	0
Corynebacterium spp	5	0	0	0

The chinchilla strains were courteously placed at our disposal by Prof. *F. Thal*, The State Veterinary Medical Institute, Stockholm.

weaker reaction with ch III and only very weak reaction with the human strain No cross reactions were obtained with ch I The *Staph aureus* strain reacted strongly with all the conjugates also with the control conjugate The Veillonella like strain reacted in the same way as the human Yersinia strain

Conjugates prepared from serum from rabbits immunized with heat killed human Y *enterocolitica* reacted less strongly with ch II and ch III than with the homologous strain The Veillonella like strain reacted as strongly as the human Yersinia strain

TABLE 2

The Reactive Properties of Different FITC Labelled Anti Yersinia enterocolitica *Globulins and Normal Rabbit Globulin with Different* Yersinia enterocolitica *Strains* Staphylococcus aureus *193 and a* Veillonella *like Faecal strain*

FITC labelled anti Yersinia enterocolitica rabbit globulin		Yersinia enterocolitica strains tested					
Rabbit immunized with live strains of	Reciprocals of globulin dilution	Human	Chinchilla type I	Chinchilla type II	Chinchilla type III	Staph aur strain 193	Veillonella like strain 1708
Human type	20	4+	0	4+	4+	4+	4+
	≥40	4+	0	4+	4+	4+	4+
Chinchilla type I	20	0	4+	0	0	4+	0
	≥40	0	4+	0	0	4+	0
Chinchilla type II	20	2+	0	4+	3+	4+	2+
	≥40	0	0	4+	2+	4+	0
Chinchilla type III	20	4+	0	4+	4+	4+	4+
	≥40	4+	0	4+	4+	4+	4+
FITC labelled anti Y enterocol O (human strain) rabbit globulin	20	4+	0	3+	3+	4+	4+
	≥40	4+	0	2+	2+	4+	4+
FITC labelled normal rabbit globulin	20	0	0	0	0	4+	0
	≥40	0	0	0	0	4+	0

Table 3 summarizes experiments in which human Yersinia conjugate was absorbed with homologous and heterologous strains respectively The data given in the table underline further the antigenic similarity between the human strain ch II ch III and the Veillonella like strain

Cross fluorescence studies with conjugated antiglobulins against human Y *enterocolitica* the Veillonella like strain 1708 and *Staph aureus* 193 against the respective strains (Table 4) showed that the two first mentioned conjugates reacted in exactly the same way The FA titres of both conjugates were 1 160 for Y *enterocolitica* and 1 320 for the Veillonella like strain The *Staph aureus* strain on the other hand showed no antigenic properties common to the other 2 strains

TABLE 3

The Reactive Properties of Unabsorbed and Absorbed FITC Labelled Anti Yersinia enterocolitica *Globulins with Different Strains of* Yersinia enterocolitica Staphylococcus aureus *193 and a* Veillonella *like Faecal Strain*

FITC labelled anti Yersinia enterocolitica (live human strain) rabbit globulin		Yersinia enterocolitica strains tested: Human	Chinchilla type I	Chinchilla type II	Chinchilla type III	Staph aur strain 193	Veillonella like strain 1708
Unabsorbed	20	4+	0	4+	4+	4+	4+
	≥40	4+	0	4+	4+	4+	4+
Abs with human	20	0	0	0	0	4+	0
O antigen	≥40	0	0	0	0	4+	0
Abs with ch I	20	4+	0	4+	4+	4+	4+
O antigen	≥40	4+	0	4+	4+	4+	4+
Abs with ch II	20	2+	0	0	1+	4+	3+
O antigen	≥40	0	0	0	0	4+	2+
Abs with ch III	20	3+	0	1+	0	4+	1+
O antigen	≥40	2+	0	0	0	4+	0
FITC labelled normal rabbit globulin	20	0	0	0	0	4+	0
	≥40	0	0	0	0	4+	0

Reciprocals of globulin dilution

TABLE 4

The Reactive Properties of FITC Labelled Anti Yersinia enterocolitica *Globulin Anti* Staphylococcus aureus *Globulin and Anti* Veillonella *like Strain Globulin with the Respective Strains*

FITC labelled rabbit anti globulins: Rabbit immunized with	Reciprocals of globulin dilution	Strains tested: Yersinia enterocolitica human strain	Staph aureus strain 193	Veillonella like strain 1708
Yersinia enterocolitica	20	4+	4+	4+
human strain live	≥40	4+	4+	4+
Staph aur strain	20	0	4+	0
193 formol killed	≥40	0	4+	0
Veillonella like	20	4+	4+	4+
strain 1708 live	≥40	4+	4+	4+
FITC labelled normal	20	0	4+	0
rabbit globulin	≥40	0	4+	0

Table 5 shows the results of attempts to eliminate the non specific bacterial fluorescence. The fluorescence from the staphylococci could be almost eliminated by absorption of the conjugate with *Staph aureus* 193 or by counterstaining with lissamine rhodamine conjugated anti staphylococcal serum. Both these methods eliminated the fluorescence also of all other staphylococcal strains tested. In the latter method the

weaker reaction with ch III and only very weak reaction with the human strain No cross reactions were obtained with ch I The *Staph aureus* strain reacted strongly with all the conjugates also with the control conjugate The Veillonella like strain reacted in the same way as the human Yersinia strain

Conjugates prepared from serum from rabbits immunized with heat killed human *Y enterocolitica* reacted less strongly with ch II and ch III than with the homologous strain The Veillonella like strain reacted as strongly as the human Yersinia strain

TABLE 2

The Reactive Properties of Different FITC Labelled Anti Yersinia enterocolitica *Globulins and Normal Rabbit Globulin with Different* Yersinia enterocolitica *Strains* Staphylococcus aureus *193 and a Veillonella like Faecal strain*

FITC labelled anti Yersinia enterocolitica rabbit globulin		Yersinia enterocolitica strains tested					
Rabbit immunized with live strains of	Reciprocals of globulin dilution	Human	Chinchilla type I	Chinchilla type II	Chinchilla type III	Staph aur strain 193	Veillonella like strain 1708
Human type	20	4+	0	4+	4+	4+	4+
	>40	4+	0	4+	4+	4+	4+
Chinchilla type I	20	0	4+	0	0	4+	0
	≥40	0	4+	0	0	4+	0
Chinchilla type II	20	2+	0	4+	3+	4+	2+
	≥40	0	0	4+	2+	4+	0
Chinchilla type III	20	4+	0	4+	4+	4+	4+
	≥40	4+	0	4+	4+	4+	4+
FITC labelled *anti Y enterocol O* (human strain) rabbit globulin	20	4+	0	3+	3+	4+	4+
	≥40	4+	0	2+	2+	4+	4+
FITC labelled normal rabbit globulin	20	0	0	0	0	4+	0
	≥40	0	0	0	0	4+	0

Table 3 summarizes experiments in which human Yersinia conjugate was absorbed with homologous and heterologous strains respectively The data given in the table underline further the antigenic similarity between the human strain ch II ch III and the Veillonella like strain

Cross fluorescence studies with conjugated antiglobulins against human *Y enterocolitica* the Veillonella like strain 1708 and *Staph aureus* 193 against the respective strains (Table 4) showed that the two first mentioned conjugates reacted in exactly the same way The FA titres of both conjugates were 1 160 for *Y enterocolitica* and 1 320 for the Veillonella like strain The *Staph aureus* strain on the other hand showed no antigenic properties common to the other 2 strains

TABLE 3

The Reactive Properties of Unabsorbed and Absorbed FITC Labelled Anti Yersinia enterocolitica Globulins with Different Strains of Yersinia enterocolitica Staphylococcus aureus 193 and a Veillonella like Faecal Strain

FITC labelled anti Yersinia enterocolitica (live human strain) rabbit globulin		Yersinia enterocolitica strains tested					
		Human	Chinchilla type I	Chinchilla type II	Chinchilla type III	Staph aur strain 193	Veillonella like strain 1708
Unabsorbed	20	4+	0	4+	4+	4+	4+
	≥40	4+	0	4+	4+	4+	4+
Abs with human	20	0	0	0	0	4+	0
O antigen	≥40	0	0	0	0	4+	0
Abs with ch I	20	4+	0	4+	4+	4+	4+
O antigen	≥40	4+	0	4+	4+	4+	4+
Abs with ch II	0	2+	0	0	1+	4+	3+
O antigen	≥40	0	0	0	0	4+	2+
Abs with ch III	20	3+	0	1+	0	4+	1+
O antigen	≥40	2+	0	0	0	4+	0
FITC labelled normal rabbit globulin	20	0	0	0	0	4+	0
	≥40	0	0	0	0	4+	0

Reciprocals of globulin dilution

TABLE 4

The Reactive Properties of FITC Labelled Anti Yersinia enterocolitica Globulin Anti Staphylococcus aureus Globulin and Anti Veillonella like Strain Globulin with the Respective Strains

FITC labelled rabbit anti globulins		Strains tested		
Rabbit immunized with	Reciprocals of globulin dilution	Yersinia enterocolitica human strain	Staph aureus strain 193	Veillonella like strain 1708
Yersinia enterocolitica	20	4+	4+	4+
human strain live	≥40	4+	4+	4+
Staph aur strain	20	0	4+	0
193 formol killed	≥40	0	4+	0
Veillonella like	20	4+	4+	4+
strain 1708 live	≥40	4+	4+	4+
FITC-labelled normal	20	0	4+	0
rabbit globulin	≥40	0	4+	0

Table 5 shows the results of attempts to eliminate the non specific bacterial fluorescence The fluorescence from the staphylococci could be almost eliminated by absorption of the conjugate with *Staph aureus* 193 or by counterstaining with lissamine rhodamine-conjugated anti staphylococcal serum Both these methods eliminated the fluorescence also of all other staphylococcal strains tested In the latter method the

specific fluorescence was retained without loss of titre The fluorescence of the Veillonella like strain could not be eliminated

Table 5 also shows that the blocking tests were of no value for distinguishing Y *enterocolitica* from the Veillonella like strain

Table 6 summarizes the results of inhibition tests with antisera against Y *enterocolitica* and the Veillonella like strain on the respective FITC-conjugated antiglobulins The results are compatible with those found previously

TABLE 5

FA Titres of FITC Labelled anti Yersinia enterocolitica *Globulin with* Y enterocolitica Staphylococcus aureus *and a Veillonella like Strain before and after Absorption with Mouse Liver and* Staph aureus *and after the Addition of Lissamine Rhodamine Labelled anti* Staph aureus *Serum*

FITC labelled anti Y enterocolitica (human live strain) rabbit globulin	Strains tested: Y enterocolitica human strain	Staph aur strain 193	Veillonella like strain 1708
Unabsorbed	160	320	320
Abs with mouse liver	40–80	80–160	160
Abs with Staph aur strain 193	40	5	160
With RB 200 labelled anti Staph aur serum added	160	≥20 buff brown red colour	320
FITC labelled normal rabbit globulin	0	160	0
Inhibition test with anti Y enterocolitica (human live strain) serum	Staining inhibited	Staining inhibited	Staining inhibited
Inhibition test with normal rabbit serum	*Staining not* inhibited	Staining inhibited	*Staining not* inhibited

Reciprocals of globulin dilution

DISCUSSION

In recent years the range of application of the FA technique has rapidly been widened Every new application involves its own problems mainly concerning specificity The present immunization procedure with *Yersinia enterocolitica* produced sera with high antibody titre enabling considerable dilution and thereby good specificity In dilution 1 10 the conjugates fluoresced more or less with several enterobacteria and streptococcal strains but already in a dilution of 1 40 practically all of the non specific fluorescence had disappeared (Table 1) The conjugates then allowed further dilution to 1 160 It was however found that even in this dilution all of the rabbit sera used reacted with cer

TABLE 6

One Step Inhibition Tests of FITC Labelled anti Yersinia enterocolitica Globulin and anti Veillonella like Strain Globulin with Y enterocolitica and the Veillonella like Strain

Conjugates	Strains tested	
	Y enterocolitica human strain	Veillonella like strain 1/08
FITC labelled anti Y enterocol (human live strain) rabbit globulin	160	320
Inhibition test with anti Y enterocolitica serum	Staining inhibited	Staining inhibited
Inhibition test with anti Veillonella like strain serum	Staining inhibited	Staining inhibited
FITC labelled anti Veillonella like strain rabbit globulin	160	320
Inhibition test with anti Y enterocolitica serum	Staining inhibited	Staining inhibited
Inhibition test with anti Veillonella like strain serum	Staining inhibited	Staining inhibited

Reciprocals of globulin dilution

tain *Staph aureus* strains Thus FITC conjugated globulins from non immunized rabbits used as control conjugates exhibited a strong fluorescence equal to that of Yersinia conjugate with these strains All the sera contained also staphylococcal agglutinins in high titre Such common occurrence of staphylococcal antibodies in normal laboratory rabbit sera has been reported earlier (*Pittman & Moody* 1960 *Cohen et al* 1961 *Bergman et al* 1966) The fluorescence of the staphylococci could be blocked by any rabbit serum It could also be eliminated by absorption of the conjugate with a staphylococcal strain (Table 5) The blocking method however produced no loss of titre and resulted also in other advantages when used in the following way To achieve maximum blocking an antistaphylococcal rabbit serum conjugated with lissamine rhodamine (RB 200) was used This was added undiluted in equal volumes to the diluted anti Yersinia-conjugate The resulting red brown fluorescence of the staphylococci and the faint background staining gave good contrast to the apple green FITC fluorescence of Y *enterocolitica* Also heterologous staphylococci and other bacteria to which antibodies spontaneously occurred in the RB 200 conjugate became contrast stained

There is a reason to believe that this counterstaining technique might be of value for the examination of clinical specimens, *e.g.* faeces, because even non bacterial faecal material including leucocytes took on the colour of the contrast stain. The method has been used successfully by *Danielsson* (1965) for demonstration of gonococci.

The examination of a number of faecal samples resulted in the isolation of a further 3 strains reacting strongly with Yersinia conjugate. Identification of these apparently identical strains which were isolated from different specimens implied certain difficulties because they were small anaerobic gramnegative cocci which grew only in extremely small colonies barely detectable with the naked eye on solid medium. So far they have refused to grow in liquid medium. Their identity is thus uncertain but in some respects they resemble Veillonella. Morphologically they were fairly easy to distinguish from Yersinia. Both in the faeces and from plate cultures they were spherical and only half as large as Yersinia. They stained intensely but did not show the marked marginal staining typical of Yersinia. They did not react with any of a number of normal rabbit globulin conjugates tried. It can be excluded that they were contaminants of the Yersinia strains used for immunization. From data summarized in Tables 2–4 it is obvious that the Veillonella like strain showed a remarkable antigenic similarity to the human *Y. enterocolitica* strain. They reacted alike in the specificity tests and could not be distinguished by the counterstaining technique. FITC conjugated antiglobulin from rabbits immunized with the Veillonella like strain likewise gave strong reactions with human *Y. enterocolitica*.

No immunological differences were observed among the 12 human *Y. enterocolitica* strains tested. Examination of one of these and of 3 of the chinchilla strains is accounted for in Tables 2 and 3. One of the chinchilla strains (ch I) deviated from the other two (ch I and ch II) having considerable antigenic similarities to the human strain. With the use of absorbed conjugates all strains could be individually differentiated to a certain extent (Table 3). The findings agree with the previous mentioned investigations by *Winblad* according to which strain ch I prosesses the O antigen factor IV, strain ch II the factors I, II and III, strain ch III the factors II and III and the human strain the factor II. The strongly deviating strain ch I was the only one among all 20 *Y. enterocolitica* strains that reacted negatively with anti *Y. enterocolitica* (human live strain) conjugate in working dilution.

SUMMARY

Antisera were obtained by immunization of rabbits with living *Yersinia enterocolitica*. The FITC labelled globulins allowed dilution to 1:160 for the staining of homologous bacterial smears.

The conjugates were tested on 20 strains of *Y. enterocolitica*, 12 of

these strains had been isolated from man and did not show any immunological differences 8 strains had been isolated from chinchilla and deviated in some respects

The high FA titre resulted in good immunological specificity according to tests with 258 non Y *enterocolitica* strains most of which were negative

The conjugate gave strong fluorescence with certain *Staph aureus* strains which proved to be due to staphylococcal antibodies normally occurring in rabbit sera This non specific fluorescence could be avoided by absorption of the conjugate with a staphylococcal strain or by blocking counterstaining with lissamine rhodamine conjugated anti staphylococcal serum The latter method has several advantages

The only cross reactions of practical importance were obtained with an anaerobic gram negative so far unidentified Veillonella like coccus found in human faeces Fluorescence immunologically it appeared to be identical with the human strains of Y *enterocolitica* It was however morphologically fairly easy to distinguish it from Yersinia No reactions were obtained with Veillonella strains isolated from throat swabs

Three of the Y *enterocolitica* strains isolated from chinchilla were examined more closely Two of these showed considerable fluorescence immunological similarities with one another and with the strains isolated from man while the third deviated strongly

It may be assumed that the FA technique especially in combination with the blocking counterstaining technique will prove useful for the demonstration of *Yersinia enterocolitica* in clinical specimens

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SEROLOGICAL STUDIES ON PROTEOLYTIC ENZYMES OF GENUS *CLOSTRIDIUM*

By

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Received 3 vi 67

Mandia (1955) found that nine recognized types of *Clostridium tetani* shared a heat stable antigenic factor IV in common with the species *Clostridium sporogenes Clostridium parabotulinum* and *Clostridium histolyticum* By absorption of the antisera with representative strains of these four species the agglutinin factor IV was removed and species specific factors labelled I II III and V remained for *Cl sporogenes*, *Cl parabotulinum Cl histolyticum* and *Cl tetani* strains respectively Similar cross reactions have been observed using agar gel diffusion technique (*Bjorklund & Berengo* 1954) and fluorescent antibody technique (*Batty & Walker* 1964 *Georgala* 1964) Thus there is as yet no complete serological survey of this genus since no definite conclusion can be drawn owing to inter species cross reactions of somatic antigens On the other hand many members of the genus *Clostridium* are characterized by serological identification of the toxins they produce Toxin production is however not a sufficiently constant character to provide a basis for classification It seems also difficult to differentiate between the closely related species on the basis of cultural biochemical and morphological properties Subdivision of the genus into species may therefore still be a matter for discussion

Sandvik (1962) has described an immunoelectrophoretic method for the serological differentiation of extracellular bacterial proteinases Most of the enzymes produced by certain aerobic and facultatively anaerobic bacteria were specific for species and cross reactions between species and genera were exceptional The serological identification of biocatalysts is considered a valuable taxonomic criterion for the differentiation of species By using suitable antigens marked by their functional enzymatic properties it is possible to identify various organisms even though the enzymes are not purified

In the present work this method was used for a further study of the serological relationship between the species of genus *Clostridium*

MATERIALS AND METHODS

Organisms.—By preliminary studies such strains of *Clostridium* as yielded the best enzymatic activities were selected. The organisms used in the present investigation isolated and classified by the Department of Microbiology and Immunology at the Veterinary College of Norway (NVH) were as follows: *Cl. perfringens* (NVH 839, NVH 18), *Cl. parasporogenes* (NVH 970), *Bacillus cereus* (NVH 5, NVH 16, NVH 132), *Bacillus subtilis* (NVH 2260), *Staphylococcus aureus* (NVH 3a5), *Pseudomonas aeruginosa* (NVH 150), *Corynebacterium pyogenes* (NVH 431), *Serratia marcescens* (NVH 119).

Strains obtained from Dr. *A. Skulberg*, the Research Committee for Preservation of Agricultural Food Products, Oslo, were: *Cl. botulinum* type A (Strain 4587), *Cl. botulinum* type B (Strain Okra), *Cl. botulinum* type E (Strain Fredriksberg).

Strains supplied by the National Collection of Type Cultures (NCTC), U.K., were: *Cl. sporogenes* (NCTC 532), *Cl. bifermentans* (NCTC 2914), *Cl. histolyticum* (NCTC 503).

Enzymes.—The various proteinases were produced by growing the clostridia in Robertson's cooked meat broth at 37° C for 2 days. Proteinases produced by *Cl. botulinum* type E were an exception, as these organisms were grown in proteose peptone—yeast extract medium at 30° C for 10 days (*Gordon et al.* 1957). Aerobic and facultatively anaerobic organisms which served as controls were grown in litmus milk medium at 37° C for 1–2 days.

Harvesting of cultures and the concentration and purification procedures have been described (*Sandvik* 1962). The liquid medium was centrifuged at approximately 25000 × g for 15–20 minutes, after which the enzyme preparation was concentrated by salting the supernatant with ammonium sulphate to 80 per cent saturation. The preparation was allowed to stand at 4° C overnight, after which the precipitate was collected by high speed centrifugation. Then the precipitate was suspended in a small volume of distilled water and dialysed under constant agitation against large quantities of tap water and subsequently distilled water. Thimerosal was added to final concentration of 1:10 000.

Sera.—Antiproteinases were produced in rabbits by subcutaneous injection of the concentrated crude enzyme mixed with equal amount of Freund's complete adjuvant (Difco).

Caseinate Medium.—The medium used to test for proteinase activity was prepared as follows: Agar (Difco Bacto agar 0140-01) 1.40 per cent, sodium caseinate[1] (added as 4.0 per cent solution of pH 7.2) 1.00 per cent, thimerosal 0.01 per cent, $MgCl_2$ (added as 10.0 per cent solution) 0.004 M in distilled water. The pH was adjusted to 6.2.

Serological differentiation.—The antiserum was electrophorized by paper electrophoresis before being brought into contact with the enzymes. A type 3276 BN LKB (Stockholm) apparatus was used with Schleicher and Schull No. 2043 bmgl paper. A 0.05 M phosphate buffer pH 6.2 with thimerosal added to a final concentration of 1:10 000 was used. The sera were added in 8–10 μl amounts and electrophorized at 120 v for 16 to 18 hours. The wet paper strips were then transferred immediately to the surface of the caseinate medium. After incubation at 37° C for 2 to 3 hours the strips were removed from the medium and replaced by narrow (0.5 to 0.8 cm) strips of filter paper which had been immersed in solutions containing the proteinase to be tested. Usually 3 to 4 filter strips were placed in parallel within the 4 cm broad field of the electrophoresis paper for 2 to 18 hours at 37° C depending on the amount of development desired. Precipitation zones occurred along the enzyme containing strips (Fig. 1). Inhibition of the zone along the application line (AP) is due to specific antienzymes in the area of the gammaglobulins. The normal inhibitors in serum were localized in the alpha and beta globulins on the anode side of the line of application.

RESULTS

All the proteolytic clostridia strains tested showed typical casein precipitating abilities (CP reaction) on caseinate agar.

[1] P914 Eastman Kodak, Rochester N.Y.

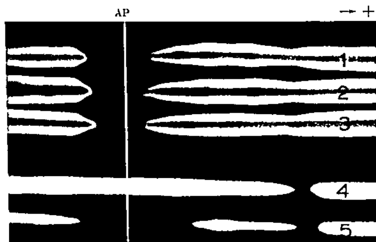


Fig 1

Electrophoretic patterns for antiserum against proteinases produced by *Cl perfringens* (NVH 839) Developments are performed with different proteolytic enzymes 1 *Cl perfringens* (NVH 839) 2 *Cl parasporogenes* (NVH 970) 3 *Cl perfringens* (NVH 18) 4 *Cl bifermentans* (NCTC 2914) 5 *Cl sporogenes* (NCTC 532) The specific antibodies are localized slightly to the left of the line of application (AP) and the normal serum inhibitors to the right The electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 hours at 120 v

The proteinases produced by two different strains of *Cl perfringens* (NVH 18 NVH 839) one strain of *Cl parasporogenes* (NVH 970) and two strains of *Bacillus cereus* (NVH 16 NVH 132) were used to stimulate antiproteinase production in rabbits In all cases good yields of antiproteinases were obtained An attempt to produce antiproteinase against *Cl bifermentans* (NCTC 2914) was unsuccessful due to high toxicity of the antigen in rabbits In Table 1 are shown the results of the serological tests when enzyme preparations from various organisms were tested with the different antiserums

The casein precipitating enzymes produced by the investigated strains of *Cl perfringens Cl parasporogenes* and *Cl sporogenes* all gave a homologous type of reaction both with the two *Cl perfringens* antienzymes and with antienzyme against *Cl parasporogenes* Similar homologous type reactions were observed when proteinases obtained from *Cl botulinum* type A (R form) type B (R form) and type E were tested with antienzymes against the two strains of *Cl perfringens* (Fig 1)

Antienzyme against *Cl parasporogenes* gave a reaction of nonidentity when tested with enzymes produced by *Cl botulinum* types A and B

It will be noticed that *Cl botulinum* types A and B proteinases differed in their reaction with antienzymes against *Cl perfringens* according as they existed in S or R form Proteinases of *Cl botulinum*

related to *Cl perfringens* (NVH 18) than to *Cl perfringens* (NVH 839) or that NVH 18 serum recognized additional antigenic determinants on the proteinase molecule as compared with the NVH 839 serum Another reason may be that two different casein precipitating enzymes are produced by *Cl histolyticum* Further investigations using purified enzymes may throw some light on this problem

Cl bifermentans (NCTC 2914) has so far not shown any relationship to *Cl perfringens* and *Cl parasporogenes*

The close relationship between some of the proteolytic clostridia is manifest but additional work with sera against *Cl bifermentans, Cl sporogenes* and *Cl botulinum* proteinases is necessary in order to get more detailed information about the enzymo serological relationship of the said species

As proteolytic enzymes from different species of genus *Clostridium* have proved to be serologically identical or closely related it is possible that transference of genetic material within these species may take place Thus a revision of the subdivision of this genus may be nec essary

SUMMARY

Proteolytic strains of genus *Clostridium* have been studied by means of serological differentiation of their proteolytic enzymes The technique is a special immunoelectrophoretic procedure in which proteolytic activity of the enzymes is neutralized by specific antiserums

A close relationship between the proteolytic strains of *Cl perfringens, Cl sporogenes* and *Cl parasporogens* was found

Cross reactions between enzymes of *Cl perfringens* and *Cl botulinum* types A B and F were observed especially when *Cl botulinum* types A and B were in R form

Cl histolyticum differed in its enzymo serological relationship to two different strains of *Cl perfringens* included in the study

Cl bifermentans did not show any serological relationship to *Cl perfringens* and *Cl parasporogenes* Genus *Clostridium* did not show any inter generic enzymo serological relationship to a number of facultatively anaerobic organisms tested

The taxonomic significance of the observed cross reactions has been discussed

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Chief R. Saxholm M D

PREVENTION OF BACTERIAL GROWTH IN URINE SAMPLES BY THE USE OF EDATHAMIL CALCIUM DISODIUM (CA TITRIPLEX)

By

KRISTIAN ÖDEGAARD

A change has taken place in recent years regarding the bact[illegible] examination of urine samples The use of a catheter for [illegible] lection of specimens has been abandoned because of the risk [illegible] ducing infection It has been replaced by clean voided midstrea[illegible] specimens where however a certain amount of bacterial c[illegible] tion especially in women is inevitable The quantitative uri[illegible] makes it possible to a great extent to distinguish between ba[illegible] tent in urine due to contamination and bacterial content d[illegible] fections in the patient (*Kass* 1955 and 1956)

Urine is an excellent medium for the growth of many b[illegible] using the quantitative method it is therefore necessary [illegible] plating carried out before the microbes have the opportu[illegible] tipling excessively This can be done in two ways either [illegible] tion immediately after urination or by chilling the urine a[illegible] it under refrigeration until it can be cultivated Both of th[illegible] are to a large extent impracticable There are few pla[illegible] possible to carry out bacteriological cultivation with a c[illegible] urine specimen and to send a urine sample to the la[illegible] refrigeration would prove expensive and time consuming [illegible]

If it were possible to find a substance which when ad[illegible] would bring about a bacteriostatic effect so that the num[illegible] bacteria remained constant this substance could then be [illegible] samples which were to be sent to a laboratory for bact[illegible] amination

We have been searching for such a substance and [illegible] series of them for their efficacy Edathamil Calcium [illegible] cium disodium salt of ethylene diaminetetra acetic a[illegible] sodium EDTA) has proved the most promising and [illegible] paper is to give an account of the tests with this sub[illegible]

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By

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Received 6 xi 67

A change has taken place in recent years regarding the bacteriological examination of urine samples The use of a catheter for routine collection of specimens has been abandoned because of the risk of introducing infection It has been replaced by clean voided midstream urine specimens where however a certain amount of bacterial contamination especially in women is inevitable The quantitative urine culture makes it possible to a great extent to distinguish between bacterial content in urine due to contamination and bacterial content due to infections in the patient (*Kass* 1955 and 1956)

Urine is an excellent medium for the growth of many bacteria and using the quantitative method it is therefore necessary to have the plating carried out before the microbes have the opportunity of multipling excessively This can be done in two ways either by inoculation immediately after urination or by chilling the urine and keeping it under refrigeration until it can be cultivated Both of these methods are to a large extent impracticable There are few places where it is possible to carry out bacteriological cultivation with a completely fresh urine specimen and to send a urine sample to the laboratory under refrigeration would prove expensive and time consuming

If it were possible to find a substance which when added to urine would bring about a bacteriostatic effect so that the number of viable bacteria remained constant this substance could then be used in urine samples which were to be sent to a laboratory for bacteriological examination

We have been searching for such a substance and have examined a series of them for their efficacy Edathamil Calcium Disodium (calcium disodium salt of ethylene diamine tetra acetic acid calcium disodium EDTA) has proved the most promising and the aim of this paper is to give an account of the tests with this substance in urine

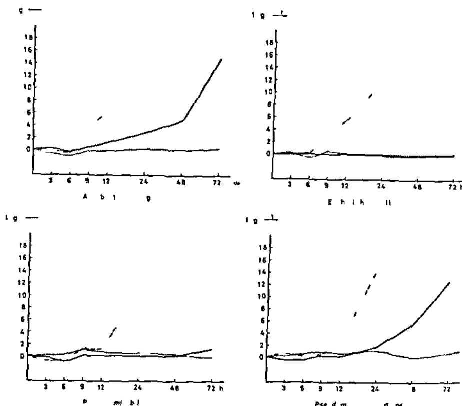


Fig 1

The growth of *Aerobacter aerogenes Escherichia coli Proteus mirabilis* and *Pseudomonas aeruginosa* in urine

N_t = the bacterial number per ml urine at the time t

N_0 = the initial bacterial number

– – – = urine at 21 C

= urine at 6 C

——— = urine with 10 per cent Edathamil Calcium Disodium and 0 01 per cent para aminobenzoic acid at 21 C

MATERIAL AND METHODS

Samples of urine were taken from three healthy individuals The samples were mixed together and filtered through a millipore GS 0 22 μ filter

In the tests which are mentioned here the pH of the urine varied between 6 and 6 5 The bacteria used were isolated in this department from urine samples from patients suspected of having urinary tract infections One strain from each of the following species was employed *Aerobacter aerogenes Escherichia coli Proteus mirabilis Pseudomonas aeruginosa Streptococcus faecalis Staphylococcus albus* and *Staphylococcus aureus*

The bacteria were spread on nutrient agar plates and incubated overnight at 3 C The colonies were suspended in nutrient broth to a density MacFarland standard No 6 and then diluted in saline and added to the urine so that the concentration of bacteria was approximately 10 000 viable bacteria per ml

Three test tubes were used for each of the strains each containing 10 ml of urine Ten per cent Edathamil Calcium Disodium [$(C_{10}H_{12}O_8N_2Ca)Na_2$ $6H_2O$] (Calcium Titriplex® rein (E Merck AG Darmstadt)) and 0 01 per cent para amino benzoic acid had previously been added to one of the test tubes The para amino

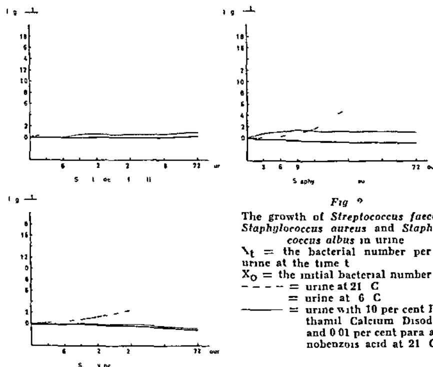


Fig 2

The growth of *Streptococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus albus* in urine

N_t = the bacterial number per ml urine at the time t

X_0 = the initial bacterial number

– – – – = urine at 21 C

= urine at 6 C

——— = urine with 10 per cent Edathamil Calcium Disodium and 0 01 per cent para aminobenzoic acid at 21 C

benzoic acid was added because this is an advantage in routine testing if a possible sulphonamide inhibition in the specimens is to be overcome The sample was then incubated at 21 C (room temperature) The two other test tubes containing urine only were incubated at 21 C and 6 C respectively

Immediately before incubation and after 3 6 9 24 48 and 72 hours incubation 1 ml of urine from each of the test tubes was diluted 100 times in saline and 1 ml of this dilution was pipetted onto a plate to which was added melted nutrient agar The plates were then incubated for approximately 20 hours after which the colonies were counted The urine samples without the additional Ca Titriplex were not tested after 48 and 72 hours incubation at 21 C due to the rate of bacterial growth, and they had therefore also to be diluted 10 000 times after 9 hours and and 1 000 000 times after 24 hours incubation to obtain countable colonies in the poured plates

RESULTS

The results can be seen in Figs 1 and 2

Each of the curves represents the averages of three tests The results show that in the main 10 per cent Ca Titriplex in the urine at room temperature (21 C) kept the number of bacteria employed in the tests almost constant for the whole of the 72 hours testing time with the exception of *Aerobacter aerogenes* and *Pseudomonas aeruginosa* where the number showed an increase The number however for both of these microbes was satisfactory after 24 hours at room temperature

DISCUSSION

The fact that urine is a good medium for the growth of several kinds of bacteria (*Asscher et al* 1966 *Aurelius* 1962) made it natural to make efforts to find an efficient method of sending the samples for quantitative bacteriological culture *Elliot & Sleigh* (1963) describe a container to be used for the transport of urine specimens at low temperature *Mackey & Sandys* (1965) employ a Dip inoculum transport medium and *Bradley et al* (1966) recommend direct inoculation of the urine onto *MacConkey* plates with swabs before the inoculated plates are sent to the laboratory It would be much simpler if a suitable substance with a bacteriostatic action instead could be added to the urine

The bacteriostatic action of Ca Titriplex is possibly the result of its affinity for some cations forming complexes in which the ions are incorporated (sequestered) in a ring structure In this way cations which are perhaps necessary for the multiplication of the bacteria may be sequestered

The growth of the bacteria in the urine is also dependent upon its pH (*Aurelius* 1962 *Asscher et al* 1966) and osmolality (*Asscher et al* 1966) and it therefore remains to be tested whether Ca Titriplex is useful if added to samples from clinical material with its variations in the pH and osmolality of the urine

It may also prove beneficial to add Ca Titriplex to specimens of water before they are sent for bacteriological examination We are investigating this possibility

SUMMARY

In the search for a substance which might have a bacteriostatic action in urine samples sent for bacteriological examination Edathamil Calcium Disodium (Ca Titriplex) has given the most satisfactory results This substance was added in a concentration of 10 per cent to sterile filtered urine which was inoculated with bacteria of seven different species which are common in urinary tract infections

This led to a satisfactory bacteriostatic action on all used bacteria after 24 hours incubation at room temperature (21 C) With regard to five of the species the result was satisfactory also after 72 hours of incubation the number of bacteria in the urine samples remaining almost constant similar to the bacterial number in the urine samples without any addition which had been kept under refrigeration

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Acta path et microbiol scandinav 71 398–408 1967

The Department of Virus Research Karolinska Institutet and the National Bacteriological Laboratory Stockholm Sweden

ANTIBODY SPECTRUM OF HUMAN GAMMA GLOBULIN FOR CLINICAL USE

By

PERNILLA MAGNUSSON and LARS OLOF KALLINGS

Received 9.1.67

Human gamma globulin is extensively used for the prevention of a large variety of infectious conditions It was considered imperative to have a better knowledge of the microbiological spectrum of antibodies present and to know the extent to which the origin of the different batches of gamma globulin influences the antibody content so as to be able to estimate the value of this administration In this study 10 batches of human gamma globulin have been tested for the presence of antibodies against an assortment of infectious agents occurring in Sweden Whether gamma globulin representing a great number of blood donors could be used for a rough epidemiological screening is also discussed

MATERIALS AND METHODS

Gamma globulin 10 *batches* of human gamma globulin each representing 5000 10 000 blood donors were obtained from *AB Kabi* Stockholm Sweden

The batches were not representative of the composition of commercial gamma globulin which is always *mixed* from various sources of raw material Four batches were prepared from retroplacental blood collected in Sweden while the others came from out dated plasma in blood banks Of these one batch came from Sweden one Finland and Sweden one Ireland and Sweden one Norway and Ireland and two originated from Bulgaria (Table 1) The gamma globulin was prepared according to a *modification* of the method of *Deutsch et al* (1946) followed by *further* purification of fraction II by *chromatography* on DEAE Sephadex

When tested by analytical ultra centrifugation the final product was found to consist of 1–3 per cent of 3–5S 85 per cent of 7S and 1 –14 per cent of 10S components No heavier components could be demonstrated

The batches were used without any addition of preservatives The materials were collected during the period between Sept *1964* and May 1965

In Sweden gamma globulin has generally been used as a 1? per cent solution In this study however only *1 per cent solutions* have been used in *order* to obtain a more direct correspondence to conventional serum titres

All dilutions of the gamma globulins were made in phosphate *buffered* saline of pH 7

As the gamma globulin preparations were *found* to have a *high anti-complementary* activity complement fixation tests had to be excluded

Virological Tests

Neutralization of 100 TCID (tissue culture *infective* doses) of *virus or haem*agglutination inhibition of 4 HAU (haemagglutinating units) of virus antigen have been used throughout Antibodies against

TABLE 1
Origin of Gamma Globulin

Gamma globulin batch no	Extracted from	Collected in
1	RPB	Sweden
2	RPB	Sweden
3	RPB	Sweden
4	MP	Norway and Ireland
5	MP	Sweden
6	MP	Sweden and Finland
7	MP	Sweden and Ireland
8	RPB	Sweden
9	MP	Bulgaria
10	MP	Bulgaria

RPB = Retroplacental blood MP = Mixed plasma of out dated blood from bloodbanks Collecting period Sept. 1964–May 1965

Polio virus type 1 strain Brunhilde type 2 strain CMRL Lansing and type 3 strain Leon were tested in primary cercopithecus monkey kidney tissue culture tubes *after binding for 6 hours at 37 C plus 12 hours at 4 C*

ECHO virus type 1 strain Farouk type 3 strain Morrissey type 4 strain du Toit type 6 strain Burgess type 7 strain Wallace type 9 strain Hill and type 11 strain Gregory were tested in primary cynomolgus monkey kidney tissue culture tubes after binding for 1 hour at 2? C (performed by Dr *T Johnsson*)

Coxsackie virus type A7 strain N 532 (State Serum Institute Copenhagen Denmark) was tested by intracerebral inoculation into new born mice (performed by Dr *Monica Grandien*) Type A9 (National Bacteriological Laboratory SBL Stockholm) and type B1 strain Conn–5 type B2 strain Ohio–1 type B3 strain Nancy type B4 strain Powers type B5 strain Faulkner and type B6 strain P?183 were tested in primary cercopithecus monkey kidney tissue tubes after binding for 2 hours at 22 C

Influenza A2 Singapore 1/57 F A2 England 1 /64 B Johannesburg 33/58 and B Singapore 3/64 were tested by inhibition of haemagglutination of rooster blood cells (performed by Dr *K E Myrback*)

Measles virus the Edmonston strain was tested by inhibition of haemagglutination (HAI) of cynomolgus monkey blood cells with Tween ether treated antigen (Norrby 1962) (performed by Dr *E Norrby*)

Parotitis virus (SBL, Stockholm) was tested by HAI of rooster blood cells (performed by Dr *E Norrby*)

Adeno virus types 2 and 4 the prototype strains were tested by HAI of rat blood cells types 3 7 and 11 by HAI of monkey blood cells according to *R sen* (1960) The test of type 2 was made more sensitive by haemagglutination enhancement by the presence of type 5 antiserum (performed by Dr *E Norrby*)

Herpes simplex virus was obtained from D A Sv dmyr and tested by neutralization in HeLa cells (performed by Dr *T Johnsson*)

Cytomegalovirus strain ad 169 obtained from Dr W P R we was tested by neutralization according to the technique outlined elsewhere (*R we et al* 1956) (performed by Dr *Gun Carlstrom*)

RSSF virus the Hypr strain was tested by neutralization in Detroit 6 cells according to techniques outlined elsewhere (*Ze pel et al* 1958) (performed by Dr *A Svedmyr*)

Vaccinia virus (SBL, Stockholm) was tested by HAI of rooster blood cells (performed by Dr *T Johnsson*)

Rubella virus strain Judith was tested by neutralization in RK13 cells after binding for 1 hour at 22 C

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The batches were used without any addition of preservatives The materials were collected during the period between Sept 1964 and May 1965

In Sweden gamma globulin has generally been used as a 12 per cent solution In this study however only *1 per cent solutions* have been used in order to obtain a more direct *correspondence to conventional serum titres*

All dilutions of the gamma globulins were made in phosphate buffered saline of pH 7

As the gamma globulin preparations were found to have a high anti complementary activity complement fixation tests had to be excluded

Virological Tests

Neutralization of 100 TCID (tissue culture infective doses) of virus or haemagglutination inhibition of 4 HAU (haemagglutinating units) of virus antigen have been used throughout Antibodies against

a result it would be interesting to trace variations in the general immunity against ECHO and Coxsackie virus infections In this study (Table 3) it has not been possible to find antibodies directed against ECHO virus types 1 3 and 4 while types 6 7 9 and 11 are fairly evenly represented

The techniques used for the determination of ECHO and Coxsackie virus antibodies have been less sensitive than those used in case of poliovirus antibodies Consequently the values found for the different groups of entero viruses are not directly comparable and the negative findings need not necessarily indicate a complete absence of antibodies According to reports on virus isolations performed between 1958 and 1965 in Swedish laboratories type 9 virus has dominated the ECHO virus statistics during the past three years Types 3 and 4 were prevalent in 1963 and in 1965 type 4 represented 50 per cent of all isolated ECHO viruses The types 1 6 and 7 have been found only in sporadic cases since 1958 and it could be questioned whether the absence of antibodies against ECHO virus types 3 and 4 in gamma globulin pools might suggest that these infections were more severe thus motivating hospitalization and virological diagnosis

TABLE 3

Neutralizing Antibodies against ECHO Viruses

Batch no	ECHO type 1	3	4	6	7	9	11
1	<1 5	<1 5	<1 5	1 5	1 5	1 5	1 5
2	<1 5	<1 5	<1 5	1 5	1 5	1 5	1 10
3	<1 5	<1 5	<1 5	1 5	1 5	1 5	1 10
4	<1 5	<1 5	<1 5	1 5	1 25	1 5	1 10
5	<1 5	<1 5	<1 5	1 25	1 5	1 5	1 10
6	<1 5	<1 5	<1 5	1 10	1 5	1 5	1 10
9	<1 5	<1 5	<1 5	1 10	1 25	1 5	1 25
10	<1 5	<1 5	<1 5	1 25	1 100	1 5	1 10

In contrast ECHO virus type 11 could be considered well represented in the population according to the gamma globulin titres in spite of the fact that only very few isolations have been done during the past 7 years On the assumption that the infectious agent is widely spread in the population it should be met with already during the first years of life probably causing only a mild infection The antibody titres recorded in this study should then represent the persisting immunity state It should also be kept in mind that before 1958 isolations of these strains were not performed routinely in Sweden The slightly higher titres of antibodies against ECHO virus type 7 in the Bulgarian batches are interesting to note

As regards Coxsackie viruses (Table 4) the results are much the same No antibodies were found against types B5 and B6 while B2 B4

A7 and A9 are generally represented In 1961 there was an epidemic in Sweden during which type B5 was isolated on more than 100 occasions but this has apparently not had any influence on the general immunity No cases involving A7 only very few involving A9 have been reported since 1958 The recent findings pertaining to Coxsackie B virus are in agreement with the results of a previous investigation (*Kallings* 1963) in which a more sensitive technique was used for comparison of the antibodies in gamma globulin batches with a screening of sera from individuals in various age groups For instance in the commercial gamma globulin preparations as usual derived from adults any antibodies against B5 were not demonstrable whereas such antibodies appeared in 46 per cent of children bled after 1961 as opposed to 6 per cent in children bled before that time

TABLE 4
Neutralizing Antibodies against Coxsackie Viruses

Batch no	B1	B2	B3	B4	B5	B6	A7	A9
1	1 5	1 5	<1 5	1 25	<1 5	<1 5	1 25	1 25
2	<1 5	1 25	<1 5	1 25	<1 5	<1 5	1 25	1 25
3	1 5	1 25	1 5	1 25	<1 5	<1 5	1 25	1 25
4	<1 5	1 5	1 5	1 25	<1 5	<1 5	1 25	1 25
5	1 5	1 5	<1 5	1 25	<1 5	<1 5	1 125	1 25
6	1 5	1 5	<1 5	1 5	<1 5	<1 5	1 25	1 100
9	<1 5	1 5	<1 5	1 25	<1 5	<1 5	1 25	1 10
10	<1 5	1 25	<1 5	1 5	<1 5	<1 5	1 25	1 10

TABLE 5
Haemagglutination Inhibiting Antibodies against Influenza Measles and Parotitis Viruses

Batch no	Influenza type A2S	A21	BJ	BS	Measles	Parotitis
1	1 32	1 16	<1 8	<1 8	1 200	1 60
2	1 32	1 16	<1 8	<1 8	1 200	1 60
3	1 32	1 32	<1 8	<1 8	1 200	1 60
4	1 32	1 16	1 8	<1 8	1 200	1 60
5	1 32	1 16	1 8	<1 8	1 200	1 60
6	1 32	1 16	1 8	<1 8	1 200	1 60
7	1 32	1 16	1 8	<1 8	1 200	1 60
8	1 32	1 16	1 8	<1 8	1 200	1 60
9	1 32	1 16	<1 8	<1 8	1 200	1 60
10	1 32	1 16	<1 8	<1 8	1 400	1 60

A2S = A2 Singapore 1571 A21 = A2 England 12/64 BJ = Johannesburg 13 58
BS = B Singapore 3/64

The antibody tests of influenza viruses reflect a quite different situation (Table 5) The individual immunity is known to be of short

duration For epidemiological reasons however a basal immunity might be expected in the population if the infections re occur with regular periodicity The A strains tested here appeared epidemically in 1960 and 1963 the titres are relatively high and show an even distribution The B strains occurred only locally and this would probably explain the lower values

Measles and parotitis virus tests (Table 5) exhibit as expected high and evenly distributed antibody titres Because of the stability of these tests and the high sensitivity obtained by Tween and ether treated HA antigen in the case of measles virus measles and parotitis viruses would provide a reliable basis for standardization purposes

Antibody titres against adenoviruses (Table 6) are dominated by type 2 followed by type 4 In contrast to this the Swedish isolation reports show a clear prevalence of type 7 with one peak in 1959 and another one in 1964 a fairly regular occurrence of type 3 and only a sparse recovery of type 2 The above indicates that adenovirus type 2 occurs mainly as a mild or subclinical infection

TABLE 6

Haemagglutination Inhibiting Antibodies against Adenoviruses

Batch no	Adenovirus type 2	3	4	7	11
1	1 240	1 30	1 30	<1 30	<1 30
2	1 480	1 60	1 60	1 30	<1 30
3	1 480	1 30	1 60	1 30	<1 30
4	1 480	1 60	1 60	1 30	<1 30
5	1 480	1 30	1 120	1 30	<1 30
6	1 480	1 30	1 120	1 30	<1 30
9	1 480	<1 30	1 120	<1 30	<1 30
10	1 240	<1 30	1 60	1 30	<1 30

TABLE 7

Neutralizing Antibodies against Herpes Simplex Cytomegalo RSSE and Rubella Viruses Haemagglutination Inhibiting Antibodies against Vaccinia Virus

Batch no	Herpes simplex	Cytomegalo virus	RSSE	Vaccinia	Rubella
1	1 125	1 25	1 [illegible]	1 [illegible]	1 5
	1 12	1 25	1 2	1 0	1 25
3	1 125	1 5	1 2	1 2[illegible]	1 2
4	1 125	1 5	[illegible]	1 20	1 5
5	1 125	1 5	1 [illegible]	1 0	1 25
6	1 125	1 5	1 2	1 20	1 125
7	1 125	<1 5	1 [illegible]	1 20	1 25
8	1 1 5	1 5	1 4	1 0	1 5
9	1 12	1 5	neg	1 0	1 25
10	1 125	1 5	neg	1 0	1 1 5

A7 and A9 are generally represented In 1961 there was an epidemic in Sweden during which type B5 was isolated on more than 400 occasions but this has apparently not had any influence on the general immunity No cases involving A7 only very few involving A9 have been reported since 1958 The recent findings pertaining to Coxsackie B virus are in agreement with the results of a previous investigation (*Kallings* 1963) in which a more sensitive technique was used for comparison of the antibodies in gamma globulin batches with a screening of sera from individuals in various age groups For instance in the commercial gamma globulin preparations as usual derived from adults any antibodies against B5 were not demonstrable whereas such antibodies appeared in 46 per cent of children bled after 1961 as opposed to 6 per cent in children bled before that time

TABLE 4

Neutralizing Antibodies against Coxsackie Viruses

Batch no	Coxsackie type B1	B2	B3	B4	B5	B6	A7	A9
1	1 5	1 [illegible]	<1 5	1 25	<1 5	<1 5	1 25	1 25
2	<1 5	1 25	<1 5	1 25	<1 5	<1 5	1 25	1 25
3	1 5	1 25	1 5	1 25	<1 5	<1 5	1 25	1 25
4	<1 5	1 [illegible]	1 5	1 25	<1 5	<1 5	1 25	1 25
5	1 5	1 5	<1 5	1 25	<1 5	<1 5	1 125	1 25
6	1 5	1 5	<1 [illegible]	1 5	<1 5	<1 5	1 25	1 100
9	<1 5	1 5	<1 [illegible]	1 25	<1 5	<1 5	1 25	1 10
10	<1 5	1 25	<1 [illegible]	1 5	<1 5	<1 [illegible]	1 25	1 10

TABLE 5

Haemagglutination Inhibiting Antibodies against Influenza Measles and Parotitis Viruses

Batch no	Influenza type A 2 S	A 2 E	BJ	BS	Measles	Parotitis
1	1 32	1 16	<1 8	<1 8	1 200	1 60
2	1 32	1 16	<1 8	<1 8	1 200	1 60
3	1 32	1 32	<1 8	<1 8	1 200	1 60
4	1 32	1 16	1 8	<1 8	1 200	1 60
5	1 32	1 16	1 8	<1 8	1 200	1 60
6	1 32	1 16	1 8	<1 8	1 200	1 60
7	1 32	1 16	1 8	<1 8	1 200	1 60
8	1 32	1 16	1 8	<1 8	1 200	1 60
9	1 32	1 16	<1 8	<1 8	1 200	1 60
10	1 32	1 16	<1 8	<1 8	1 400	1 60

A2S = A2 Singapore 1/57 F A2E = A2 England 12/64 BJ = Johannesburg 33/58 BS = B Singapore 3/64

The antibody tests of influenza viruses reflect a quite different situation (Table 5) The individual immunity is known to be of short

duration For epidemiological reasons however a basal immunity might be expected in the population if the infections re-occur with regular periodicity The A strains tested here appeared epidemically in 1960 and 1963 the titres are relatively high and show an even distribution The B strains occurred only locally and this would probably explain the lower values

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Antibody titres against adenoviruses (Table 6) are dominated by type 2 followed by type 4 In contrast to this the Swedish isolation reports show a clear prevalence of type 7 with one peak in 1959 and another one in 1964 a fairly regular occurrence of type 3 and only a sparse recovery of type 2 The above indicates that adenovirus type 2 occurs mainly as a mild or subclinical infection

TABLE 6
Haemagglutination Inhibiting Antibodies against Adenoviruses

Batch no	Adenovirus type 2	3	4	7	11
1	1 240	1 30	1 30	<1 30	<1 30
2	1 480	1 60	1 60	1 30	<1 30
3	1 480	1 30	1 60	1 30	<1 30
4	1 480	1 60	1 60	1 30	<1 30
5	1 480	1 30	1 120	1 30	<1 30
6	1 480	1 30	1 120	1 30	<1 30
9	1 480	<1 30	1 120	<1 30	<1 30
10	1 240	<1 30	1 60	1 30	<1 30

TABLE 7
Neutralizing Antibodies against Herpes Simplex Cytomegalo RSSE and Rubella Viruses Haemagglutination Inhibiting Antibodies against Vaccinia Virus

Batch no	Herpes Simplex	Cytomegalo virus	RSSE	Vaccinia	Rubella
1	1 125	1 2	1 2	1 20	1 5
2	1 125	1 5	1 2	1 0	1 25
3	1 125	1	1 2	1 0	1 25
4	1 125	1	neg	1 20	1 5
5	1 125	1	1	1 20	1 25
6	1 1	1	1	1 20	1 125
7	1 1	<1	1	1 20	1
8	1 12	1	1 4	1 20	[illegible]
9	1 1	1	neg	1 20	[illegible]
10	1 1	1	n	1 0	[illegible]

Titres against herpes simplex virus (Table 7) are quite comparable to convalescent values. According to our present knowledge, however, the administration of gamma globulin does not prevent infections caused by the herpes group of viruses.

As yet the true frequency of cytomegalovirus (Table 7) is not known. According to the findings of antibodies in the gamma globulin batches, however, it should be a fairly common infection.

RSSE virus has been isolated from meningitis patients in Sweden on several occasions, varying from 10 to 30 per year. No antibodies could be detected in the batches from Bulgaria, Norway or Ireland (Table 7), while a low titre was found constantly in the batches originating in material obtained in Sweden. RSSE virus infections occur principally along the east coast of Sweden (*v. Zeipel et al.* 1959), but unfortunately it is not known from which parts of the country the blood materials originated.

Vaccinia virus antibodies are constant throughout the batches (Table 7). The titres correspond to those found in individuals who have been vaccinated.

The rubella virus antibody titres are high but variable (Table 7). It should be stressed again that the materials were tested as 1 per cent solutions. *Schiff et al.* (1963) have tested Swedish immuno globulin prepared from rubella convalescent sera as 12 per cent solution against 1:10 of the virus concentration used in this study. The antibody titres were found to range from 1:512 to 1:4000. With respect to the differences in the methods, the values of normal gamma globulin are quite comparable to those of the immuno globulins.

Bacteriological Tests

Antistreptolysin titres equal the average values for normal healthy persons (Table 8) and also antistaphylolysin titres fall within the normal range (Table 8). By and large, the results of the other bacteriological tests carried out are meagre. It is not astonishing to find that the Widal test is negative against Typhus H antigen in the Scandinavian batches, as the corresponding infections occur very infrequently in Scandinavia. Anticolilysin is seldom found in younger healthy persons (Table 8). Antipneumolysin normally ranges between 50 and 100. The values found here are slightly lower (Table 8).

Demonstrable antitetanus titres were present in the 1 per cent gamma globulin solutions, while antidiphteria titres could only be found in 10 per cent solutions. It should be observed that the lowest titres of both tests are found in gamma globulin stemming from retroplacental blood. Swedish men are given booster doses of a vaccine containing tetanus and diphteria toxoids during their military service and this fact probably accounts for the differences found here.

Antibodies to *Mycoplasma pneumoniae* could be measured in all

batches tested (Table 9) However the tetrazoleum reduction inhibition test has been used only recently and as serological screenings with this test are still scarce the evaluation of the results presented here will have to await further information

TABLE 8

Antibodies against Some Bacterial Antigens

Batch no	AS	ASTA	Widal	ACL	APL	Diphtherias	Tetanus
1	100	1 5	<1 40	≤70	50	0 7	>0 01–<0 1
2	100	0 7	<1 40	≤70	50	0 7	>0 01–<0 1
3	100	0 5	<1 40	≤70	50	0 7	>0 1 –<1 0
4	100	0 5	<1 40	≤70	≤36	8	>0 1 –<1 0
5	70	1 0	<1 40	≤70	≤36	4 5	>0 1 –<1 0
6	70	0 7	<1 40	≤70	≤36	4	>0 1 –<1 0
7	N T †	N T	N T	≤70	≤36	4	>0 1 –<1 0
8	N T	N T	N T	≤70	≤36	0 5	>0 1 –<1 0
9	100	0 36	1 40	≤70	≤36	4	>0 1 –<1 0
10	70	0 7	1 40	≤70	≤36	4	>0 1 –<1 0

AS = Antistreptolysin ASTA = Antistaphylolysin ACL = Anticolilysin APL = Antipneumolysin Antitetanus and Antidiphtheria titres given as I U of Antitoxin S typhi H antigen (d)

§ Tested as 10 per cent solutions of gamma globulin

† N T = not tested

TABLE 9

Antibodies against M Pneumoniae and Toxoplasma

Batch no	Mycoplasma pneumoniae	Toxoplasma
1	1 4	1 50
2	1 4	1 50
3	1 4	1 50
4	1 4	1 50
5	1 4	1 50
6	1 4	1 50
7	1 8	1 50
8	1 2	1 50
9	1 4	1 50
10	1 4	1 50

Protozoan Test

Antibodies against toxoplasma were found in all batches The titres correspond to those of normal healthy persons who have once passed a toxoplasma infection

CONCLUSION

Judging from the presence of circulating antibodies gamma globulin administration might prevent a series of virus diseases especially in case of epidemics when incubation might be predicted and an early

administration is possible In the case of some infections i e measles the prophylactic effect has been well established by clinical experience Gamma globulin should be of use also to prevent some Coxsackie and adenovirus infections In the same way the results should confirm the effect of gamma globulin in individuals who for different reasons can not be vaccinated e g, against poliomyelitis It should also be possible to provide a protection against other enteroviruses to patients with lowered resistance to infections

In some other diseases e g parotitis where humoral antibodies are not considered to be of major importance the prophylactic value of gamma globulin may be disputable even though the batches tested have been found to contain high antibody levels

Resistance to influenza virus infections is probably mediated by antibodies mainly located to the oral and bronchial mucosa (*Smith et al* 1966) and gamma globulin administration should therefore be expected to be without effect on the attack rate

The bacterial antibody tests applied in this study failed to establish the clinical value of gamma globulin in the same clear cut manner as has been done in the case of many virus infections This is related to the limitations of the available methods but probably also to the fact that conventional gamma globulin preparations for clinical use do not contain any IgM

It is evident that a follow up of the antibody spectrum of normal human gamma globulin would be a valuable complement to the reports of virus isolations The findings in this study of antibodies against different enteroviruses reveal epidemiological patterns which could not be predicted merely on the basis of isolation reports A comparison between the isolation and serological results makes it possible to evaluate the pathogenicity of the different strains of the enteroviruses tested In this way it might be possible to trace changes in their frequency and clinical appearance

A follow up would also yield a convenient method to be used for a screening of the immunity state of the population as reflected by blood donors and parturients especially with a view to checking the results of various vaccination programmes The figures presented here concerning antibody titres against poliovirus (Table 2) and vaccinia virus (Table 7) show that the general resistance to these agents can be considered satisfactory not only for the individual but also as regards a limitation of spread of these agents in a society

SUMMARY

Ten batches of pre commercial normal human gamma globulin have been examined for antibody titres against a variety of infectious agents occurring in Sweden The batches originated mainly from Sweden but some came also from Bulgaria Finland Ireland and Norway

Antibody tests for different viruses show as expected high titres against e g polio measles and parotitis variations between the batches were only slight while antibodies against rubella were constantly found although with variable titres Furthermore antibodies were regularly demonstrated also against a series of viruses considered to occur less frequently

The findings of antibodies against different entero and adenoviruses revealed epidemiological patterns which could not be predicted merely on the basis of reports on virus isolations

As regards bacteria findings cannot be expected to be positive to the same extent as in the case of viruses taking into consideration the limitations of the methods used and the fact that conventional gamma globulin preparations for clinical use do not contain IgM For some bacterial antigens however *e g* strepto and staphylolysin antibodies were demonstrated in amounts corresponding to the average level found in healthy individuals

The results of vaccination against for instance polio and variola as well as diphteria and tetanus were clearly reflected in the tests

It is evident that a follow up of the antibody spectrum of normal human gamma globulin would yield a convenient method to be used for a screening of the immunity state of the population as reflected by blood donors and parturients

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A follow-up would also yield a convenient method to be used for a screening of the immunity state of the population as reflected by blood donors and parturients, especially with a view to checking the results of various vaccination programmes. The figures presented here concerning antibody titres against poliovirus (Table 2) and vaccinia virus (Table 7) show that the general resistance to these agents can be considered satisfactory not only for the individual but also as regards a limitation of spread of these agents in a society.

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IMMUNOCHEMICAL CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* CELL WALLS

By

ARNE GROV and SIDSEL RUDE

Received 16 vi 67

Most antigenic studies on isolated *Staph aureus* cell walls have been performed on walls which may have been exposed to the action of cellular enzymes or have been treated with enzymes Walls which are the object of immunological studies should as far as possible be undigested Irrespective of the method used for isolation the structure of *Staph aureus* cell walls has been found to be composed of two major components *i e* ribitol teichoic acid and mucopeptide (glycopeptide) Various serological reactions given by *Staph aureus* cells and extracts of these have been demonstrated with wall preparations as well *i e* agglutination precipitation and sensitization of tanned sheep erythrocytes (23 18 17 15) *Stern & Elel* (23) found that undigested cell wall preparations from *Cowan* s three types of *Staph aureus* possessed the same agglutinogens as whole cells The precipitating ability of teichoic acid has been established (11 3) *Juergens et al* (17) concluded after specific inhibition studies that the teichoic acid was also responsible for the agglutination of the cell walls and in addition *Morse* (18) ascribed the sensitizing ability to this component Neither *Juergens et al* nor *Morse* could demonstrate serological activity in isolated mucopeptide Recently the sensitizing ability has been shown to be due to the mucopeptide (21 4) and furthermore a precipitating polypeptide has been isolated from this component (13)

The present paper deals with preparation of undigested *Staph aureus* cell walls and investigation of their antigenic properties

MATERIALS AND METHODS

Strains The *Staph aureus* type strains C[illegible] an I and 1503 were selected for preparation of cell walls Both strains are ri h n the precipitinogens protein A and polysaccharide A and contain subst n whi h sensitize tanned and normal sheep erythrocytes to agglutin tion Th n t pe agglutinogens of strain Cowan I are

The authors are indebted t D F I *Selwig* for performing the electron microscopic examinations.

h_2 l_1k_2 m 263-1 and 263-2 and those of strain 1503 are e m and n The n antigen also produces a line on agar gel diffusion

Growth and harvesting The bacteria used for preparation of cell walls were grown for 18 hrs on nutrient agar plates and were harvested by scraping the surfaces The Cowan I bacteria used for testing the h_2 and k_1l_2 factor sera were grown for 18 hrs on mannitol salt agar

Preparation of cell walls The procedure employed for preparation of cell walls was that recommended by Yoshida et al (24) involving disintegration of cells in the X press at approx −25 C washing of the disintegrated cells several times with phosphate citrate buffer and M KCl followed by density gradient and pile centrifugations The prepared cell walls were dialysed and freeze dried The purity of the preparations was controlled by chromatographic examination on nucleic acid components in 0 1 N HCl hydrolysates and by electron microscopy In the electron microscopic study single drops of homogenized suspensions of cell walls as well as of whole cells were placed on specimen grids previously covered with carbon substrates and allowed dry The specimens were shadowed with palladium from an angle of approx 20 and subsequently examined in the electron microscope (Siemens Elmiskop I)

Acid hydrolysis Portions of cell walls were hydrolysed in 0 1 N HCl for 2 hrs at 100 C in 1 N HCl for 3 hrs at 100 C in 6 N HCl for 18 hrs at 105 C and in 2 N H_2SO_4 for 3 hrs at 100 C The hydrolysate were evaporated to dryness in vacuo taken up in measured volumes of water and subjected to qualitative circular paper chromatographic analysis according to the method described earlier (5) An additional solvent system the organic phase of BuOH EtOH H_2O NH_3 d 0 88 (40 10 49 1 v/v) (1) was employed for the identification of sugar alcohols

Analytical methods Determination of nitrogen phosphorus and hexosamines was performed according to the methods referred to in (5)

Antigen preparations The protein A and polysaccharide A (strain Wood 46) preparations used in this study were described in (3) and (4) respectively

Antisera Rabbit antisera against whole cells were produced as described by Oeding (20) The same technique was used for production of antisera against isolated cell walls except that the walls were not pre treated with formalin Cell walls were suspended in saline at a concentration of 1 mg per ml and a total of 8 3 mg of freeze dried cell wall was injected into each rabbit

Factor e serum was prepared as proposed by Haukenes & Oeding (12) factor h_2 l k_2 m and b_1 sera according to Haukenes (8 9 10) and factor n and 263 1 sera according to Hofstad (14)

Serological Methods Bacterial agglutination was performed on slides as described by Oeding (19) and *cell wall agglutination* in tubes essentially according to Morse (18) For cell wall agglutination the sera were diluted serially tenfold and 0 25 ml of the serum dilutions was added to small test tubes (13×85 mm) To each tube was then added one drop of a cell wall suspension (2 mg per ml saline) which had previously been homogenized by ultrasonics for 10 to 15 mins Readings were made after incubation at 37 C for 30 mins and overnight at room temperature

Ring test agar gel precipitation and *indirect haemagglutination* were carried out as in previous experiments (5)

RESULTS

The cell wall preparations were white in colour The electron micrographs showed the typical picture of cell wall preparations mainly consisting of empty shells and with very few if any whole cells present When 0 25 mg of cell walls hydrolysed in 0 1 N HCl was applied to the paper on chromatography no spots related to nucleic acids were detected by examination of the dried chromatogram under a UV lamp One μg of adenine and 1 μg of guanine gave brightly fluorescent bands The quantitative analytical data are listed in Table 1

Chromatographic examination of 3 N HCl hydrolysates and of 2 N H_2SO_4 hydrolysates revealed identical components Cell walls of both

strains contained glucosamine muramic acid and ribitol Glycerol aldohexoses aldopentoses and uronic acids could not be observed

In 6 N HCl hydrolysates 10 amino acids were demonstrated (Table 1) No qualitative difference in the amino acid content of the two cell wall preparations could be observed

TABLE 1

Results of Quantitative and Chromatographic Analyses of the Cell Wall Preparations

	Cell wall of strain	
	1503	Cowan I
Phosphorus per cent	1 88	1 75
Nitrogen per cent	8 50	9 80
Glucosamine (free base) per cent	13 50	11 50
Muramic acid	++	++
Ribitol	+++	+++
Lysine	+++	+++
Aspartic acid	++	++
Serine	++	++
Glycine	+++	+++
Glutamic acid	+++	+++
Alanine	+++	+++
Threonine	(+)	(+)
Proline	+	+
Valine	+	+
Leucine	+	+

(+) to +++ Trace to strong reaction on chromatograms with the respective spray reagents

The results of most of the serological experiments are summarized in Table 2 No antigen *n* line was observed with 1503 cell wall in the agar precipitation test Of the fractions obtained by the preparation procedure only the cytoplasmic fraction produced the *n* line However absorption of *n* factor serum with 1503 cell wall removed the agglutinins

Polysaccharide A precipitins could not be demonstrated in the cell wall antisera whereas both antisera contained antibodies against protein A (precipitation titres in agar 1 16 Furthermore the 1503 cell wall antiserum gave a weak *n* line against extract of whole 1503 bacteria but no *n* line against suspension of 1503 cell wall

Cell walls of both strains agglutinated in the 17 *Staph aureus* type sera tested and in the two cell wall antisera With the exception of strain Wood 46 in Cowan I cell wall antiserum the two cell wall antisera agglutinated all of the 17 *Staph aureus* type strains

No agglutination was obtained with 1503 cell wall (6 mg per ml) in *e m* and *n* factor sera or with Cowan I cell wall in *h*, k_1k_2, *m* and *963-1* factor sera Attempts to prepare *m* factor serum from Cowan I cell wall antiserum and *e m* and *n* factor sera from 1503 cell wall anti

h_2 $l_1 k_2$ m 263-1 and 263-2 and those of strain 1503 are e m and n The n antigen also produces a line on agar gel diffusion

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tein A and with the two cell wall preparations On the other hand tanned erythrocytes sensitized with protein A preparation removed corresponding agglutinins only Removal of agglutinins to sensitized tanned erythrocytes did not reduce the precipitation or bacterial agglutination titres of any of the sera Neither was the bacterial agglutination titre of antiserum to whole cells affected by absorption of precipitins to polysaccharide A whereas a significant reduction was observed on absorption of diluted serum (1 40) with the protein A preparation

DISCUSSION

During the procedure employed for preparation the cell walls were exposed to cellular enzymes for only a short time at 0 C and may be considered as undigested The electron microscopic pictures and the chromatographic examination on nucleic acid components indicated satisfactorily pure cell wall preparations

Chemical analyses of the cell walls revealed the same amino acids as found in protein A (5) Of these lysine glycine glutamic acid and alanine are regularly found in walls of *Staph aureus* strains (22) but small amounts of aspartic acid serine threonine valine and leucine have also been reported (16 7 2 15) To our knowledge however proline has not previously been demonstrated in *Staph aureus* cell wall preparations Protein A containing proline (5) is digestible by trypsin The other components demonstrated *i e* organic phosphorus ribitol glucosamine and muramic acid are integral parts of *Staph aureus* cell walls (22)

The cell wall preparations were serologically active in the agar gel precipitation test in agglutination reactions and as sensitizing agents in the indirect haemagglutination test The precipitinogens polysaccharide A and protein A have also previously been shown to be cell wall components (11 25 15) Whereas the *n* antigen could not be demonstrated in 1503 cell wall by agar precipitation or by agglutination in *n* factor serum the cell wall absorbed *n* agglutinins Furthermore the 1503 cell wall antiserum produced a weak *n* line against extract of whole 1503 bacteria and contained small amounts of *n* agglutinins Although very little *n* antigen is likely to be present in our cell wall preparation it must be regarded as a wall component

All *Staph aureus* antisera tested agglutinated both cell wall preparations but no agglutination was obtained in factor sera The presence of type agglutinogens in the cell wall preparations was however demonstrated by the removal of agglutinins from factor sera by absorption The failure of agglutination in factor sera may be due to the low antibody content of these sera and the apparently small amounts of type agglutinogens present in the walls The use of ultrasonics before the agglutination test may have caused a further extraction

The cell wall antisera agglutinated both cell wall preparations and

all but one of 17 *Staph aureus* strains Except for a weak *n* factor serum all attempts to prepare factor sera from the cell wall antisera were unsuccessful Similar findings were made by *Hofstad* (15) in experiments with several cell wall antisera The absence of type agglutinins in cell wall antisera or their presence in only small amounts may be due to the small amounts of corresponding antigens in the wall preparations but may also be due to the immunization technique employed The absence of antibodies against the teichoic acid in cell wall antisera may also be ascribed to the immunization technique or to the possibility of structural changes during preparation of the walls

A suspension of Cowan I cell wall sensitized normal and tanned sheep erythrocytes to agglutination in antisera against whole bacteria and in Cowan I cell wall antiserum This shows that sensitizing antigens are located in the wall That 1503 cell wall lacked the antigen which sensitized normal sheep erythrocytes indicates not only a difference in composition but also structural variations in the two wall preparations The antigen in 1503 bacteria that sensitizes normal sheep erythrocytes is apparently removed during preparation of the cell wall and cannot therefore be so firmly attached to the wall as the corresponding antigen in Cowan I bacteria

Complete absorption of cell wall antisera and antisera against whole bacteria of antibodies to tanned sheep erythrocytes sensitized with the polysaccharide A preparation did not reduce the precipitation or bacterial agglutination titres Neither was the bacterial agglutination titre of antiserum against whole bacteria affected by complete absorption of polysaccharide A precipitins The precipitating agglutinating and sensitizing antigens are thus different each inducing production of specific antibodies

The rigid *Staph aureus* cell wall mucopeptide (glycopeptide) has been shown to contain 4 amino acids only *i e* lysine glycine glutamic acid and alanine Since our cell wall preparations contain 6 more amino acids additional structures to those regularly found in *Staph aureus* cell walls are likely to be present This suggestion is supported by the various antigenic activities demonstrated

SUMMARY

Undigested cell walls of the *Staph aureus* strains Cowan I and 1503 have been prepared

Chemical analyses revealed the presence of organic phosphorus ribitol glucosamine muramic acid and the amino acids lysine aspartic acid serine glycine glutamic acid alanine and smaller amounts of threonine proline valine and leucine

The precipitinogens protein A and polysaccharide A were both shown to be cell wall constituents and most likely the *n* antigen as well The wall preparations sensitized tanned sheep erythrocytes one of them

also normal erythrocytes Furthermore they agglutinated in all immune sera tested and exhausted most factor sera for agglutinins by absorption It is concluded that the sensitizing antigens as well as the agglutinogens are wall components

Immune sera against the cell wall preparations contained antibodies against protein A the *n* antigen the sensitizing antigens and the non specific agglutinogens but not against polysaccharide A or most of the specific agglutinogens

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all but one of 17 *Staph aureus* strains Except for a weak *n* factor serum all attempts to prepare factor sera from the cell wall antisera were unsuccessful Similar findings were made by *Hofstad* (15) in experiments with several cell wall antisera The absence of type agglutinins in cell wall antisera or their presence in only small amounts may be due to the small amounts of corresponding antigens in the wall preparations but may also be due to the immunization technique employed The absence of antibodies against the teichoic acid in cell wall antisera may also be ascribed to the immunization technique or to the possibility of structural changes during preparation of the walls

A suspension of Cowan I cell wall sensitized normal and tanned sheep erythrocytes to agglutination in antisera against whole bacteria and in Cowan I cell wall antiserum This shows that sensitizing antigens are located in the wall That 1503 cell wall lacked the antigen which sensitized normal sheep erythrocytes indicates not only a difference in composition but also structural variations in the two wall preparations The antigen in 1503 bacteria that sensitizes normal sheep erythrocytes is apparently removed during preparation of the cell wall and cannot therefore be so firmly attached to the wall as the corresponding antigen in Cowan I bacteria

Complete absorption of cell wall antisera and antisera against whole bacteria of antibodies to tanned sheep erythrocytes sensitized with the polysaccharide A preparation did not reduce the precipitation or bacterial agglutination titres Neither was the bacterial agglutination titre of antiserum against whole bacteria affected by complete absorption of polysaccharide A precipitins The precipitating agglutinating and sensitizing antigens are thus different each inducing production of specific antibodies

The rigid *Staph aureus* cell wall mucopeptide (glycopeptide) has been shown to contain 4 amino acids only *i e* lysine glycine glutamic acid and alanine Since our cell wall preparations contain 6 more amino acids additional structures to those regularly found in *Staph aureus* cell walls are likely to be present This suggestion is supported by the various antigenic activities demonstrated

SUMMARY

Undigested cell walls of the *Staph aureus* strains Cowan I and 1503 have been prepared

Chemical analyses revealed the presence of organic phosphorus ribitol glucosamine muramic acid and the amino acids lysine aspartic acid serine glycine glutamic acid alanine and smaller amounts of threonine proline valine and leucine

The precipitinogens protein A and polysaccharide A were both shown to be cell wall constituents and most likely the *n* antigen as well The wall preparations sensitized tanned sheep erythrocytes one of them

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IMMUNOCHEMICAL EXAMINATION OF PHENYLHYDRAZINE TREATED *STAPHYLOCOCCUS AUREUS* CELL WALLS

By

ARNE GROV and SIDSEL RUDE

Received 16 vi 67

In a previous paper (6) the preparation and immunochemical properties of undigested cell walls of the *Staph aureus* strains Cowan I and 1503 were studied The precipitinogens agglutinogens and sensitizing antigens found in whole bacteria were also demonstrated in the cell wall preparations Except for polysaccharide A precipitins and most of the type agglutinins the cell wall antisera contained antibodies to all antigens demonstrated

In addition to the components regularly found in *Staph aureus* cell walls our preparations contained 6 more amino acids aspartic acid serine threonine proline valine and leucine Since isolated teichoic acid has been shown to contain ester linked alanine and the rigid mucopeptide (glycopeptide) contains 4 amino acids only (lysine glycine glutamic acid and alanine) it was assumed that our wall preparations contain additional structures This assumption was supported by the various antigenic activities demonstrated

In the present work teichoic acid has been extracted and chemical and serological examinations have been made of the extracted and the unextractable material

MATERIALS AND METHODS

Preparations The preparations employed in the present investigation i e cell wall of *Staph aureus* type strain Cowan I protein A and polysaccharide A are the same as in the previous experiments (6)

Antisera The rabbit antisera described in (6) were used

Extraction of teichoic acid Incubation of polysaccharide A with 5 per cent trichloro acetic acid for 15 mins at 90 C ([illegible] ulted in almost complete loss of serological activity by ring test Since incubation of polysaccharide A with phenylhydrazine hydrochloride (pH 7 0) at 80 C for 30 mins was shown not to reduce the ring test titre this method of extraction was preferred

The cell wall preparation was extracted with phenylhydrazine according to the description of *Archibald & Baddiley* (1) The extraction process was controlled by agar gel diffusion with polysaccharide A (teichoic acid) as indicator The combined supernatants containing teichoic acid and the unextractable residue were dialysed and freeze-dried.

All chemical and serological methods employed in this study were the same as those described or referred to in (6)

RESULTS

After two consecutive extractions of a sample of cell wall with phenyl hydrazine at 80° C for 30 mins no polysaccharide A line could be demonstrated with the washed cell wall residue by agar diffusion The results of chromatographic and serologic analyses are listed in Table 1 and 2 respectively

TABLE 1

Results of Chromatographic Analysis of Fractions of Cowan I Cell Wall

	Fractions			
	A	B	C	D
Phosphorus	+++	+++	+	+
Glucosamine	+++	+++	+++	+++
Muramic acid	—	—	++	++
Ribitol	+++	+++	—	—
Lysine	++	—	+++	+++
Aspartic acid	++	—	++	++
Serine	++	—	++	++
Glycine	++	—	+++	+++
Glutamic acid	++	—	+++	+++
Alanine	++	(+)	+++	+++
Threonine	(+)	—	(+)	(+)
Proline	+	—	+	(+)
Valine	+	—	+	+
Leucine	+	—	+	+

A = Crude phenylhydrazine extract
B = A from which protein A has been removed
C = Washed cell wall residue after phenylhydrazine extraction
D = C further extracted with phosphate buffer (pH 7 4) (protein A precipitinogen removed)
(+) to +++ Trace to strong reaction with colour reagents

TABLE 2

Results of Serological Tests Performed with Antisera and Fractions of Cowan I Cell Wall

	A	B	C	D
Agar precipitation lines with *Staph aureus* antisera	Poly A Prot A	Poly A	Prot A	No line
Agglutination in antisera against				
15 *Staph aureus* strains			+	+
1503 cell wall			+	+
Cowan I cell wall			+	+
Indirect haemagglutination in Staph aureus antisera	SNSC— STSC+	SNSC— STSC—	SNSC+ STSC+	SNSC+ STSC+

A B C, D see Table 1
SNSC = Sensitized *normal sheep cells*   STSC = Sensitized tanned sheep cells

Chromatographic examination of 3 N HCl hydrolysates of the cell wall residue revealed both glucosamine and muramic acid whereas neither ribitol nor anhydroribitol could be demonstrated Quantitative determinations of phosphorus and glucosamine (as free base) gave 0 25 and 7 9 per cent respectively

In 6 N HCl hydrolysates of the residue the same amino acids as found in hydrolysates of unextracted wall (6) were demonstrated The content of proline had however decreased relative to the other amino acids Examination of HCl hydrolysates of extracted material showed that it contained glucosamine ribitol and the same amino acids as demonstrated in unextracted wall but no muramic acid

Serologically the phenylhydrazine extract was found to contain polysaccharide A (teichoic acid) and protein A precipitinogens In addition the extract sensitized tanned sheep erythrocytes to agglutination in Cowan I immune serum By absorption the extract removed all precipitins from Cowan I immune serum and from Cowan I cell wall immune serum whereas no absorption of bacterial agglutinins could be observed even at high dilutions of the antisera

Separation of the precipitinogens protein A and polysaccharide A (teichoic acid) was obtained by precipitating protein A with HCl at pH 3 5 and precipitation of polysaccharide A from the supernatant with 4 volumes of alcohol at pH 5 2 The final alcohol precipitate after three successive precipitations with acid and alcohol produced on agar diffusion the polysaccharide A line only and showed no sensitizing ability

TABLE 3

Agglutination Titres of 3 Staph aureus Antisera with Intact Cowan I Cell Wall and Its Unextractable Material

	Intact cell wall	Unextractable material
Antiserum to strain		
Cowan I	2560	51?0
1503	1280	51?0
?095	1?80	?560

Reciprocal value of the highest dilution of antiserum which produced agglutination

Suspension of the cell wall residue gave a weak protein A line on agar diffusion and sensitized normal and tanned sheep erythrocytes to agglutination in Cowan I immune serum and Cowan I cell wall immune serum On tube agglutination the cell wall residue agglutinated in all of 15 *Staph aureus* antisera and also in the two cell wall antisera No reduction of the agglutinability could be demonstrated compared to that of unextracted cell wall (Table 3) Prolonged extraction with phosphate buffer (pH 7 4) removed protein A precipitinogen from the cell wall residue whereas the sensitizing and agglutinating activities remained Acid hydrolysates of the resultant unextractable material were

shown by chromatographic examination to contain glucosamine muramic acid, and the same amino acids is found in intact cell wall Proline and threonine were however present only in trace amounts The term unextractable *material* is used as this material contains more amino acids than the *rigid* cell wall mucopeptide (glycopeptide)

DISCUSSION

Extraction of staphylococcal cell walls with phenylhydrazine is a useful method of separating teichoic acid and mucopeptide Removal of teichoic acid was indicated by the absence of polysaccharide A line on agar diffusion of the cell wall residue The absence of ribitol in this material confirmed the serological results The amount of unextractable *phosphorus is of the same order as in previous findings* (2 3) Since no muramic acid could be demonstrated in the phenylhydrazine extract this material is likely to be free of mucopeptide

In addition to all polysaccharide A (teichoic acid) most of the protein A precipitinogen was extracted by *phenylhydrazine* The extract also sensitized tanned sheep erythrocytes but after separation of protein A and teichoic acid the sensitizing ability was found to follow protein A Absorption experiments with the phenylhydrazine extract showed that this did not affect the bacterial agglutination indicating that the agglutinogens are associated with the mucopepide component

The protein A precipitinogen remaining in the cell wall residue could be removed by extraction with a neutral phosphate buffer This indicates that protein A is loosely bound to *Staph aureus* cell walls Since it can easily be separated from teichoic acid attachment or adhesion to the mucopeptide in intact cells is most likely

The unextractable cell wall residue was agglutinated in the same sera as intact Cowan I cell wall showing that the agglutinogens belong to this fraction which is mostly made up of mucopeptide

In accordance with our previous suggestion (1) cell wall residue that is free of teichoic acid is found to sensitize tanned sheep erythrocytes In addition the unextractable material of Cowan I cell wall sensitizes *normal erythrocytes The substance in protein A preparations* which sensitizes tanned erythrocytes has been shown to be a peptide (5) while the sensitizing ability of the polysaccharide A preparation is due to the *mucopeptide* component (8 1) Whether or not the amino sugars of the mucopeptide are involved in this serological activity is not clear Since tanned erythrocytes sensitized with protein A do not adsorb antibodies to tanned erythrocytes sensitized with polysaccharide A (8 6) it is likely that the two sensitizing substances give rise to different antibodies The chemical basis for the sensitization of *normal sheep erythrocytes* is more obscure

Compared to the mucopeptide moiety of polysaccharide A preparations obtained from autolysates of whole cells the unextractable ma

terial isolated is apparently more complex Chemically the difference is clearly demonstrated in the amino acid content as the mucopeptide of polysaccharide A (strain Wood 46) contains only 5 amino acids i e lysine glycine glutamic acid serine and alanine Serologically the polysaccharide A preparation lacks the ability to inhibit bacterial ag glutination of antisera

SUMMARY

Undigested cell wall of the *Staph aureus* strain Cowan I has been ex tracted with phenylhydrazine

Teichoic acid (polysaccharide A) was completely extracted and also most of the protein A precipitinogen Additional extraction with phos phate buffer removed the rest of the protein A whereas the agglutinat ing and most of the sensitizing abilities remained in the unextractable material This material was shown to contain organic phosphorus glucosamine muramic acid lysine aspartic acid serine glycine gluta mic acid alanine smaller amounts of valine and leucine and traces of proline and threonine

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Acta path et microbiol scandinav 71 432–438 1967

Statens Seruminstitut Department of Diagnostic Bacteriology Copenhagen Denmark

STAPHYLOCOCCUS AUREUS STRAINS ISOLATED IN DANISH HOSPITALS

2 *From January 1st, 1961, to December 31st 1965*

By

KIRSTEN ROSENDAL and PER BÜLOW

Received 14 vi 67

Phage typing of *Staphylococcus aureus* strains (coagulase positive staphylococci) is performed in only one laboratory in Denmark—Statens Seruminstitut

Therefore it has been possible since April 1960 to register all phage typed staphylococci from patients and staff members of hospitals all over the country

From the very beginning of the registration it was realized that an evaluation of the frequency of hospital infections could not be carried out on the basis of the material investigated or the clinical information sent from the hospitals to the laboratory

However various aspects of the material seemed to make it valuable in other respects

The great number of strains registered (about 65 000) offered an opportunity of correlating bacteriological properties of the staphylococci even among groups of strains not frequently encountered Furthermore the fact that the strains were collected from a whole country over a long period made it possible to decide whether the constellation of the properties was a constant feature or not moreover epidemic changes of the staphylococcal population in the course of the years would necessarily be made clear from the registration

The introduction of a new experimental phage called 6557 was due to such a change during 1962 the number of non typable strains increased and their characteristics changed they were more frequently multiple resistant and a higher proportion than in the previous years were unable to produce a Tween 80 splitting enzyme (TW –) (*Bulow & Rosendal* 1964) As phage 6557 was found capable of lysing the majority of strains with these properties it was used along with the basic set of internationally recommended phages (*Blair & Williams* 1961) Strains lysed by this phage were registered separately as type 6557 and a report of their occurrence and properties is given in this paper A special detailed description of the phage the properties of

strains lysed by it and their epidemiological occurrence is to be published by *Bulow* (1968 a b c)

A survey of the material collected during the first nine months of registration has already been published (*Rosendal et al* 1963) It is the aim of the present report to give the results of the investigations of the last five years from January 1st 1961 to December 31st 1965

METHODS

The methods are the same as those described in a previous publication (*Rosendal et al* 1963) However during the five year period some minor alterations have been introduced

Registration Punch cards were used only for the registration of the material from 1961 For the last four years it was found desirable to divide the material into more subgroups and this was done more conveniently by extracting the information required directly from the original cards Therefore when the calculations were based on this subdivision the material from 1961 was omitted

Bacteriophage typing Since April 1st 1963 an experimental phage named 6557" (*Bülow & Rosendal* 1964) was added to the basic set of typing phages (*Blair & Williams* 1961)

Resistance to antibiotics has been determined in several laboratories using methods mentioned by *Rosendal et al* (1963) but since November 1961 Statens Seruminstitut has introduced a prediffusion method as described by *Thomsen* (1962 1964) The antibiotics examined are penicillin (P) streptomycin (S) tetracyclines (T) chloramphenicol (C) and erythromycin (E)

Resistance to mercuric chloride Since February 1st 1962 all phage typed strains were examined by the method given by *Moore* (1960) as described by *Jessen et al* (1963)

Production of lipase was demonstrated as described in 1963 (*Rosendal et al*) using a Tween" 80/calcium agar (*Sierra* 1957)

RESULTS

From Table 1 it is seen that the material consists of 66 219 strains (isolated from 66 219 persons) 58 419 of which have been examined for resistance to antibiotics

TABLE 1
The Material

Year	Number	Examined for resistance to antibiotics	Sens to all antibi ti s	per cent Resistant to P	S	T	C	F	TW— %	Hg+ %
1961	12003	10443	–	66	7	13	3	1	25	–
1962	12403	10987	30	60	35	17	3	2	26	38
1963	12600	10940	6	71	37	23	4	5	33	44
1964	14400	12793	24	3	35	5	4	7	29	41
1965	14813	13 6	25	2	32	27	4	10	31	39
Total	66219	58419								

Sens = sensitive P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol F = eryth omycin TW— = do n t produce the "Tween" 80 splitting enzyme Hg+ = re st nt to mercuri chloride – = not examined

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1963	12600	10940	26	71	37	23	4	5	33	44
1964	14400	1[illegible]43	24	[illegible]3	35	25	4	7	29	41
1965	14813	1[illegible]6	25	72	3[illegible]	27	4	10	31	39
Total	6[illegible] 19	[illegible]419								

Sens = sensitive P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E = erythromycin TW— = do not produce the "Tween" 80 splitting enzyme Hg+ = resistant to mercuric chloride – = not examined

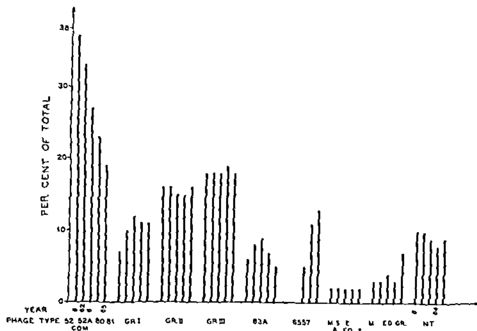


Fig 1
Percentage distribution of phage types/groups NT = non typable
Phage 6557 is used for the nine last months of 1963 only

The number of strains registered increases steadily over the years thus the material from 1965 exceeds that from 1961 by about 23 per cent

A decrease in the percentage of strains sensitive to all antibiotics is followed by a moderate increase in strains resistant to penicillin whereas the number of strains resistant to tetracyclines and erythromycin has increased considerably The percentage of strains resistant to streptomycin decreases during the years whereas that of strains resistant to chloramphenicol is fairly constant

Strains which did not produce the Tween splitting enzyme (TW—) were more frequently isolated in 1965 than in 1961 Strains resistant to mercuric chloride (Hg+) were found with about the same frequency in 1962 and 1965 However in 1963 the percentages of both TW— and Hg+ strains are higher than in 1965

Phage Types/Groups of the Strains

Fig 1 shows the percentage distribution of the phage types/groups during the years

Only minor deviations are seen in the percentage occurrence of strains belonging to groups II (15 16) III (18–19) and miscellaneous (2) The most striking variations in frequency are noticed among strains belonging to the 52 52A 80 81 complex type 83A strains and

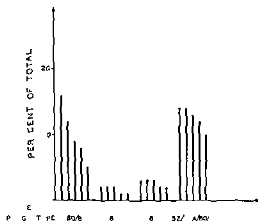


Fig 2

Percentage distribution of various phage types within the 52 52A 80 81 complex 52/52A/80/81 comprises all variations of the phage pattern 52 52A 80 81 except 80/81 80 and 81

strains lysed by phage 6557 For strains in the inhomogeneous mixed group the frequency has increased from 3 per cent in 1961 to 7 per cent in 1965 and among group I strains from 7 to 12 per cent

The 52 52A 80 81 complex which has decreased steadily from 37 per cent to 19 per cent is still the most frequently encountered group Fig 2 shows that the steepest decrease—from 16 to 5 per cent—is found among strains of type 80/81 whereas the 52/52A/80/81 strains in the same period have only fallen from 13 to 10 per cent thus being the most frequently represented of the complex The two types 80 and 81 are found more rarely their percentages in the whole material are now 1 and 2 respectively whereas the corresponding percentages in 1961 were 2 and 3

Types 83A and 6557 are closely related It is thought that the latter has originated from 83A by lysogenization with a prophage which makes it resistant to typing phage 83A (*Bulow & Rosendal* 1964 *Bulow* to be published) Fig 1 shows that type 83A culminates in 1963 (9 per cent) when phage 6557 was first utilized afterwards it decreases to 5 per cent in 1965 In the same period type 6557 increases from 5 to 13 per cent However phage 6557 was not used routinely until April 1st 1963 so it seems justifiable to assume that the percentage of this type was 7 in 1963 These hypothetical two per cent (Fig 1) can only be a matter of estimation as the strains were discarded but 47 bacteraemia strains retyped and lysed by phage 6557 at RTD had previously been registered as non typable (22) or typed at 1000×RTD as 83A (13) group III (9) and miscellaneous (3)

The percentage of the non typable strains is fairly constant but the curve would have shown a peak in 1963 if half of the 6557 strains had been added to the non typable ones registered here

Clinical Sources and the Distribution of Phage Types/Groups

The processes and the regions from which the staphylococci were isolated are given in Table 2

An examination of the whole material showed that the contribution of strains from some sources varied throughout the years (upper part) whereas the supply from other sources was fairly constant (lower part) Thus the percentage of staphylococci from abscesses and furunculi shows the most striking variation 20 in 1961 but only 8 in 1965 In the same period staphylococci from urine and faeces as well as from bronchii and sputum were registered with increasing frequency where as the percentage of specimens forwarded without satisfactory information did not vary very much

TABLE 2
Clinical Sources of Strains Investigated

Source	Percentage of total material				
	1961	1962	1963	1964	1965
Abscesses and pustuli	20	12	11	11	8
Bronchii and sputum	8	9	9	11	12
Urine and faeces	5	5	7	7	9
Not specified	29	36	32	35	33

	1961	1963
Cicatrices and wounds	8	10
Mastitis	1	1
Hands	6	4
Eye	2	1
Ear	5	4
Nose throat and sinus	9	9
Larynx and trachea	2	4
Miscellaneous§	5	5

Forwarded without information necessary for classification

§ "Miscellaneous includes specimens from blood osteomyelit viscera exudate from viscera joint fistulae from ossa and viscera vagina uret a spinal fluids burns umbilicus eczema and skin diseases and autopsy

The staphylococci from bronchii and sputum cicatrices and wounds urine and faeces nose and throat and abscesses showed the highest percentages in 1965 An intermediate group was composed of staphylococci from hands ear and larynx and trachea whereas only relatively few specimens from mastitis cases and eyes were received

Table 3 shows how frequently the various phage types/groups were found among specimens from the most numerous or important (trachea) groups of sources The figures from 1961 are compared with those from 1965 In abscesses and furunculi strains of the 52 52A 80 81 complex are still the ones most frequently isolated but the percentages of this group are decreasing in all the sources referred to The

TABLE 3

Percentage Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources from the Years 1961 and 1965

Source	Phage type/group													
	52 52A 80 81		Group I		Group II		Group III		83A		6557		NT	
	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965
Nose throat sinus	22	12	9	14	20	23	23	21	6	3	–	6	12	9
Trachea larynx	14	12	9	13	20	14	28	21	13	5	–	14	12	14
Abscesses and furunculi	5	34	7	12	15	21	10	11	2	1	–	6	7	7
Cicatrices and wounds	31	15	6	9	12	11	23	19	12	5	–	21	11	11
Bronchii and sputum	3	13	5	9	16	13	25	22	13	10	–	15	11	10
Urine and faeces	3	9	4	6	13	9	29	21	12	12	–	21	12	13
Percentage of the total material	36	18	7	11	16	16	18	18	6	5	–	13	10	9

– = not investigated. NT = non typable

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Abscesses and pustuli	20	12	11	11	8
Bronchii and sputum	8	9	9	11	12
Urine and faeces	5	5	7	7	9
Not specified	29	36	32	35	33

	1961	1965
Cicatrices and wounds	8	10
Mastitis	1	1
Hands	6	4
Eye	2	1
Ear	5	4
Nose throat and sinus	9	9
Larynx and trachea	2	4
Miscellaneous§	5	5

Forwarded without information necessary for classification

§ Miscellaneous includes specimens from blood osteomyelit' viscera exudate from viscera joint fistulae from ossa and viscera vagina uret a spinal fluids burns umbilicus eczema and skin diseases and autopsy

The staphylococci from bronchii and sputum cicatrices and wounds urine and faeces nose and throat and abscesses showed the highest percentages in 1965 An intermediate group was composed of staphylococci from hands ear and larynx and trachea whereas only relatively few specimens from mastitis cases and eyes were received

Table 3 shows how frequently the various phage types/groups were found among specimens from the most numerous or important (trachea) groups of sources The figures from 1961 are compared with those from 1965 In abscesses and furunculi strains of the 52 52A 80 81 complex are still the ones most frequently isolated but the percentages of this group are decreasing in all the sources referred to The

TABLE 3

Percentage Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources from the Years 1961 and 1965

Source	Phage type/group													
	52 52A 80 81		Group I		Group II		Group III		83A		6557"		NT	
	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965	1961	1965
Nose throat sinus	22	12	9	14	20	23	23	21	6	3	–	6	12	9
Trachea larynx	14	12	9	13	20	14	28	21	13	5	–	14	12	14
Abscesses and furunculi	55	34	7	12	15	21	10	11	2	1	–	6	7	7
Cicatrice and wounds	31	15	6	9	12	11	23	19	12	5	–	21	11	11
Bronchii and sputum	23	13	5	9	16	13	25	22	13	10	–	15	11	10
Urine and faeces	23	9	4	6	13	9	29	21	12	12	–	21	12	13
Percentage of the total material	36	18	7	11	16	16	18	18	6	5	–	13	10	9

– = not investigated. NT = non typable

percentage of group I strains is increasing in the whole material whereas that of group II strains is decreasing except among strains from abscesses and nose where they make up the most numerous group. Group III strains are less frequently isolated except among strains from abscesses. The percentage of 83A strains shows a decreasing tendency except among specimens from urine and faeces. As the new type 6557 is assumed to have originated from type 83A these two types are considered together in Table 4 where the figures from 1965 are compared with those from 1964 (the first year in which the phage 6557 was utilized throughout). It is found that the total percentage applying to these two years is identical and that strains of the phage pattern 83A/6557 are the most numerous among strains from cicatrices bronchii and urine but that the percentage among strains from trachea and cicatrices is decreasing.

TABLE 4
Combined Percentage Distribution of Phage Types 83A and 6557 among Strains from Various Clinical Sources from the Years 1964 and 1965

Source	1964	1965
Nose throat sinus	10	9
Trachea larynx	*26*	*19*
Abscesses and furunculi	8	7
Cicatrices and wounds	32	26
Bronchii and sputum	25	25
Urine and faeces	32	33
Total	18	18

Strains from these two latter sources may be of special epidemiological interest since most specimens from trachea and cicatrices are forwarded from surgical departments or intensive care units—where cross infections frequently occur and where new phage types such as 83A (*Rosendal & Jessen* 1964) and 6557 (*Bulow* 1968c) were first isolated in Denmark.

Therefore the percentage distribution of phage types among strains from trachea and cicatrices was further examined. The figures in Table 5 indicate the difference between the percentages found in 1964 and 1965. It is obvious that non typable strains and strains from mixed groups are represented in larger numbers in 1965 than in 1964. Furthermore the 52 52A 80 81 complex and strains from group I are more numerous in 1965 among strains from trachea and strains from group II among strains from cicatrices. It might be deduced from these facts that one or more "new" strains—for the time being non typable—are spreading.

If a comparison is drawn between the non typable strains seen in

1961 and 1965 (Table 3) it appears that they are isolated more frequently from specimens from trachea and less frequently from the nose in 1965 than in 1961 As far as the other clinical sources are concerned the figures applying to these two years are about the same

TABLE 5
Increase (+) or Decrease (—) of Percentage Contribution of the Various Phage Types/Groups The Material from 1964 Compared with That from 1965

Phage types/groups	Clinical sources	
	Trachea and larynx	Cicatrices and wounds
52 52A 80 81	+2	—4
Group I	+5	0
Group II	—3	+2
Group III	—1	—1
83A + 6557	—7	—6
Miscellaneous	—1	+1
Mixed group	+2	+3
NT (non typable)	+2	+4

Clinical Sources and Antibiotic Sensitivity of the Strains

From Table 1 it was obvious that the percentage of strains resistant to P T and E had increased over the years whereas that of strains resistant to S and C had not Therefore only the resistance to the former antibiotics was correlated to the clinical source of the strain (Table 6) and it is seen that in 1965 the strains most frequently resistant to these antibiotics are found among specimens from trachea cicatrices and urine

Correlation of Various Properties of the Strains

Phage types/groups and resistance to antibiotics The figures in Table 7 give the percentages of the various phage types/groups sensitive to all antibiotics examined or resistant to various combinations of antibiotics for the years 1962–1965 (It is not possible to give the corresponding percentages for 1961 as resistance to the various antibiotics in that year was registered separately) Less than 10 per cent of the strains are not recorded here namely strains in which resistance to penicillin was combined with resistance to other antibiotics in rare combination and strains sensitive to penicillin but resistant to one or more of the other antibiotics

Generally speaking the percentage of sensitive strains within the phage groups is decreasing whereas that of strains resistant to penicillin is increasing More strains of the 52 52A 80 81 complex and more non typable strains have become resistant to P+S+T and th

TABLE 6

Resistance to Penicillin Tetracyclines and Erythromycin among Strains from Various Sources

% of strains resistant to	Clinical sources: nose throat sinus 1961	1965	trachea larynx 1961	1965	abscesses furunculi 1961	1965	cicatrices wounds 1961	1965	bronchii sputum 1961	1965	urine faeces 1961	1965	Total 1961	1965
Penicillin	66	64	64	82	66	71	69	79	66	79	63	76	66	72
Tetracyclines	10	13	35	38	9	17	21	41	23	33	20	48	13	27
Erythromycin	1	4	1	14	<1	5	1	18	1	9	1	15	1	10

TABLE 7

Percentage Occurrence of Various Antibiograms within Phage Types/Groups

Phage types/groups	P 1962	P 1963	P 1964	P 1965	P + S 1962	P + S 1963	P + S 1964	P + S 1965	P + S + T 1962	P + S + T 1963	P + S + T 1964	P + S + T 1965	P + S + T + E 1962	P + S + T + E 1963	P + S + T + E 1964	P + S + T + E 1965	C 1962	C 1963	C 1964	C 1965	Sensitive 1962	Sensitive 1963	Sensitive 1964	Sensitive 1965
52/52A/80/81	18	20	20	23	52	50	45	39	6	9	10	12	0.2	0.4	0.6	1.1	2	2	3	2	13	15	16	18
Group I	34	38	44	42	3	4	3	2	2	3	2	2	0	0.4	0.3	1.2	2	3	1	3	57	51	46	46
Group II	43	41	50	54	2	2	1	1	1	1	1	1	0	0.1	0.2	0.2	2	2	2	2	46	41	37	40
42B/47C	8	14	7	14	2	8	6	9	20	14	13	16	1	0	0	3	4	3	6	10	52	59	63	43
Group III	52	57	58	60	5	4	4	3	7	9	7	6	0.4	2	4	5	4	4	5	4	24	18	16	15
83A	7	10	10	10	3	2	3	4	66	70	70	62	7	5	6	4	4	6	5	6	5	4	3	5
6/57	–	5	10	11	–	4	2	2	–	30	32	32	–	43	34	35	–	10	10	10	–	6	6	4
187	13	11	15	10	1	1	2	1	0	2	2	0	0	0	0	0	1	3	4	1	83	78	73	85
Miscellaneous	22	51	9	48	6	2	0.4	4	2	0	0.4	2	0	2	0	0	2	9	2	0	66	18	48	48
Mixed group	46	44	54	48	4	4	3	4	1	5	3	3	0.3	1	2	3	6	7	4	3	40	34	29	33
NT	25	22	32	26	5	4	4	3	10	16	10	17	9	11	6	12	3	4	4	4	40	38	38	33

Include all strains resistant to C whether they are resistant to other antibiotic(s) or not

P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E= erythromycin NT = *non typable* – = not examined

frequency of strains resistant to P+S+T+E is increasing especially among group III strains and non typable strains moreover the latter group and type 6557 contain the highest percentage of strains resistant to erythromycin

Furthermore it is noticed that a certain resistance pattern is characteristic of certain phage groups groups I and II are mostly either sensitive or resistant to penicillin only Strains belonging to the 52 52A 80 81 complex are very often resistant to P+S 62–70 per cent of type 83A are resistant to P+S+T type 6557 is frequently resistant to P+S+T (30–32 per cent) and P+S+T+E (35–43 per cent) and 10 per cent of this type are invariably resistant to C Within each phage group the percentage of chloramphenicol resistant strains does not vary much from year to year

Lipase production and mercury resistance correlated with phage type and resistance to antibiotics When the total material was examined it was found that lipase production and resistance to mercuric chloride could both be correlated with resistance to antibiotics (Table 8 bottom line) the percentage of TW— strains and mercury resistant strains was lowest among the antibiotic sensitive strains and increased with increasing multiple resistance to antibiotics being highest among strains resistant to P+S+T+E

Therefore the material was divided up according to antibiotic resistance and further subdivided according to phage types/groups (Table 8) It is seen that the tendency demonstrated in the bottom line is also found within the single phage group However a few deviations from the common pattern are noticed

Occurrence of TW — strains Previous investigations of the 52 52A 80 81 complex have shown that temperate phages blocking the sensitivity to typing phages were often able at the same time to convert Tween positivity to Tween negativity (*Rosendal et al* 1964 *Rosendal & Bulow* 1965) This is also indicated by the findings here types 80 and 81 are more frequently TW — than other members of the 52 52A 80 81 complex and many other phage typ /groups within groups of identical resistance pattern The percentage of TW — strains among types 80/81 and 52/52A/80/81 is everywhere below average whereas that among types 83A and 6557 is above average except among strains resistant to P+S+T+E where the percentage of TW — 83A strains is below average Among strains belonging to groups I and II the percentages do not increase considerably with increasing multiple resistance and are on a lower level than the other comparable strains However group II includes various types known to differ as to lipase production (*Jessen et al* 195) The group was therefore divided into types (Table 9) and the results confirm previous findings on that type 71 is far more frequently TW — than any other member of the group (*Parker* 1958) and any other phage type (phage type 80 excepted) of the same antibiotic resistance

TABLE 6

Resistance to Penicillin Tetracyclines and Erythromycin among Strains from Various Sources

% of strains resistant to	nose throat sinus 1961	1965	trachea larynx 1961	1965	abscesses furunculi 1961	1965	Clinical sources: cicatrices wounds 1961	1965	bronchii sputum 1961	1965	urine faeces 1961	1965	Total 1961	1965
Penicillin	66	64	64	82	66	71	69	79	66	79	63	76	66	72
Tetracyclines	10	13	35	38	9	17	21	41	23	33	20	48	13	27
Erythromycin	3	4	1	14	<1	5	1	18	1	9	1	15	1	10

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Phage types/groups	I 1962	1963	1964	1965	P + S 1962	1963	1964	1965	P + S + T 1962	1963	1964	1965	P + S + T + C 1962	1963	1964	1965	C 1962	1963	1964	1965	Sensitive 1962	1963	1964	1965
52 52A 80 81	18	20	20	23	52	60	45	39	6	9	10	12	0.2	0.4	0.6	1.1	2	2	3	2	13	15	16	18
Group I	34	38	44	42	3	4	3	2	2	3	2	2	0	0.4	0.3	1.2	2	3	1	3	57	51	46	46
Group II	43	41	56	54	2	2	1	1	1	1	1	1	0	0.1	0.2	0.2	2	2	2	2	46	41	37	40
42B/47C	8	14	7	14	2	8	6	9	20	14	13	16	1	0	0	3	4	3	6	10	52	59	63	43
Group III	52	57	58	60	5	4	4	3	[illegible]	9	7	6	0.4	2	4	5	4	4	5	4	24	18	16	15
83A	7	10	10	16	3	2	3	4	66	70	70	62	7	5	6	4	4	6	5	6	5	4	3	5
C557	–	5	10	11	–	4	2	2	–	30	32	32	–	43	34	35	–	10	0	10	–	6	6	4
187	13	11	15	10	1	1	2	1	0	2	2	0	0	0	0	0	1	3	4	1	83	78	73	85
Miscellaneous	22	51	9	48	6	2	0.4	4	2	0	0.4	2	0	2	0	0	2	9	2	0	66	18	48	48
Mixed group	46	44	54	48	4	4	3	4	1	5	3	3	0.3	1	2	3	6	7	4	3	40	34	29	33
NT	5	22	32	26	5	4	4	3	10	16	10	17	9	11	6	12	3	4	4	4	40	38	38	33

Includes all strains resistant to C whether they are resistant to other antibiotic(s) or not
P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E = erythromycin NT = non typable – = not examined

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TABLE 8

Percentage Occurrence of TW— and Hg+ Strains within the Various Phage Types/Groups
The Material Has Been Divided into Groups of Identical Resistance to Antibiotics
The Material Comprises the Strains from 1962–1965

Phage types/groups	Sensitive total 12399			P total 16104			PS total 6243			PST total 5886			PSTE total 2308		
	% of total	TW— %	Hg+ %	% of total	TW— %	Hg+ %	% of total	TW— %	Hg+ %	% of total	TW— %	Hg+ %	% of total	TW— %	Hg+ %
80/81	1	9	58	6	7	85	32	20	83	5	15	94	1	(19)	(89)
80	<1	20	8	<1	57	65	5	89	91	<1	(70)	(100)	<1	(50)	(100)
81	3	15	8	1	35	58	7	42	84	2	42	86	<1	(33)	(100)
52/52A/80/81	10	12	9	6	14	71	40	17	83	9	13	91	2	14	94
42B/47C	2	17	6	1	10	15	<1	(25)	(66)	1	(61)	(91)	<1	(33)	(67)
Group I	21	12	5	13	14	7	3	24	48	2	31	67	1	(67)	(80)
Group II	25	12	7	24	13	7	2	16	10	1	(21)	(35)	<1	0	(55)
Group III	13	21	7	30	25	10	5	41	51	11	56	82	10	89	94
83A	1	33	14	2	25	15	1	(51)	(86)	37	73	91	8	50	97
6557	2	20	10	2	35	15	1	(80)	(85)	20	79	97	58	97	98
Miscellaneous + 187	5	3	9	1	4	24	<1	(6)	(32)	<1	(13)	(25)	0	0	0
Mixed group	5	13	7	6	17	8	1	(26)	(54)	1	(42)	(85)	2	93	88
NT (non typable)	13	22	8	7	41	27	3	39	65	10	66	89	18	95	97
Total %		15	8		19	19		27	79		61	90		89	97

( ) = because of the small number of strains the percentages given are not reliable
P = penicillin S = streptomycin T = tetracyclines E = Erythromycin

Occurrence of Hg+ strains. Among sensitive strains and strains resistant to P only the percentage of 80/81 strains resistant to mercuric chloride differs remarkably from that of other strains (Table 8). [illegible]8 per cent of the antibiotic sensitive 80/81 strains are mercury resistant as compared with the average of 8 per cent. Among the penicillin resistant strains (P) the other members of the 52, 52A, 80, 81 complex are also more frequently resistant to mercuric chloride than the average (19 per cent), but they never reach the frequency of type 80/81 ([illegible] per cent). In group I and especially in group II the mercury resistant strains are rare as compared with the average.

TABLE 9

Percentage of TW — Strains among Strains Belonging to Phage Group II

Phage type/pattern	Sens	P res
3A	6 (814)	9 (1560)
71	40 ([illegible]89)	4[illegible] (24[illegible])
71 included in the pattern	13 (1085)	17 (1030)
Rest of group II	8 (909)	6 (1137)

Total number of strains is given in brackets.
Sens = sensitive to all antibiotics. P res = resistant to penicillin only.
Strains resistant to P+S, P+S+T, P+S+T+E are too few to allow a subdivision of the phage group.

TABLE 10

Mercury Resistance Correlated with Lipase Production

Resistance to antibiotics	Percentage of mercury resistant strains: TW — = P_1	TW + = P	$P_1 - P_2$	$3 \times \sigma_D$
Sensitive	9.8 (1809)	7.2 (10590)	2.6	[illegible]
P	21.5 (3137)	17.8 (12967)	3.7	2.43
PS	8[illegible] (1658)	77.4 (4585)	4.8	3.30
PST	91.5 (3576)	87.7 (2310)	3.8	2.4[illegible]
PSTE	98.2 (2056)	85.3 ([illegible])	12.9	6.8

Total number of strains is given in brackets.
σ_D = the standard error of the difference.
P = penicillin. S = streptomycin. T = tetracyclines. E = erythromycin.

Mercury resistance correlated with production of the "Tween" splitting enzyme. Table 10 shows that the proportion of Hg+ strains is higher among TW — strains than among TW + strains within groups of identical resistance pattern. The preponderance however does not seem to be very marked, but the large number of strains permits a statistical evaluation according to which the percentages in the two groups differ significantly, the observed differences being more than three times the magnitude of the standard error.

TABLE 11

Mercury Resistance Correlated with Lipase Production among Group III Strains

Resistance to antibiotics	Percentage of mercury resistant strains			
	TW— = P_1	TW + = P	$P_1 - P_2$	3 × εD
Sensitive	5 7 (331)	7 3 (1931)	1 6	4 6
P	15 6 (1227)	7 9 (3653)	7 7	3 3
PS	*65 3 (138)*	*41 3 (201)*	*24 0*	*16 0*
PST	89 0 (356)	73 7 (281)	15 3	9 4
PSTF	97 0 (196)	69 2 (26)	27 8	2 5

Total number of strains is given in brackets
εD = the standard error of the difference
P = penicillin S = streptomycin T = tetracyclines F = erythromycin

The same results are obtained within the single but probably in homogenous phage group III (Table 11) among the penicillin resistant (P PS PST) strains Among the TW— strains resistant to PSTF the percentage of mercury resistant is 97 whereas it is much lower (69) among the corresponding 26 TW + strains but there is no difference between the percentages in the two groups of sensitive strains

DISCUSSION

In the previous report from 1963 (*Rosendal et al*) it was mentioned that strains resistant to tetracyclines and erythromycin were less numerous in the Danish material than among strains isolated in other countries

However if the present material is compared with other materials collected outside Denmark (*Jansson & Wager* 1963 *Fowler et al* 1963 *Froman et al* 1964 *Bauer* 1965) it seems as if there is no such difference even though the materials concerned are smaller than the Danish ones and collected from more restricted areas In Denmark it was shown that the increase in the percentage of strains resistant to tetracyclines and erythromycin is primarily due to the spread of strains belonging to types 83A (*Rosendal & Jessen* 1964) and 6/57 (*Bulow* 1968c)

Fluctuations in the staphylococcal population similar to those found in the Danish material are also observed in other countries Increase in the percentage of multiple resistant strains is a common feature (*Bauer et al* 1960 *Krynski et al* 1966) from Australia (*Rountree et al* 1960) U S A (*Cohen et al* 1962) and Poland (*Krynski et al* 1966) it is reported that the incidences caused by strains of the 52 52A 80 81 complex are decreasing and that a multiple resistant epidemically occurring strain is introduced A strain with similar characteristics is described in reports from Canada (*Comtois & Bynoe* 1963) and the U K (*Temple & Blackburn* 1963) where according to *Jevons & Parker*

(1964) it apparently first appeared in 1960. In this paper as well as in a later one (*Jevons et al.* 1966) the origin of the new type is discussed. The authors conclude that it may have arisen from various strains including type 83A by lysogenization with various phages. The common characters were in most cases lysis by the experimental phages 77Ad and B5 (later introduced into the basic set of typing phages as 84 and 85), Tween negativity and resistance to neomycin.

"Type 6557 probably corresponds to the new type. Resistance to neomycin has not been registered in this material, but in a collection of about 1600 bacteraemia strains from 1957–1965 84 out of 93 neomycin resistant strains belonged to type 6557. Furthermore the majority of type 6557 are TW — and lysed by phages 84 and 85. However recent investigations have shown that methicillin resistant strains are never lysed by all three phages. The majority of them are either lysed by 6557 or 84 only; of the remaining ones the majority are non typable with an inhibition reaction caused by phage 84 at 1000× RTD. This indicates that it will not be possible to omit phage 6557 in Danish investigations and also that a new type resistant to methicillin is making its appearance.

Authors in other countries have often correlated the emergence and spread of strains resistant to neomycin with the consumption of this antibiotic (*Quie et al.* 1960, *Cohen et al.* 1962, *Mitchell* 1964, *Lowbury et al.* 1964, *Lavine et al.* 1965, *Rountree & Beard* 1965). In Denmark the appearance of type 6557 may well be connected in some way with the use of erythromycin and neomycin. Further studies concerning the development of resistance to these antibiotics will be published later by *Bulow*.

The size of the material made it possible to correlate resistance to mercuric chloride with phage types of identical resistance to antibiotics. Thus phage type 80/81 was found to be characteristic in that it was resistant more frequently than other types (*Moore* 1960, *Richmond & John* 1964). This seems to support the concept of a connexion between communicability and mercury resistance. However the present decrease in the incidences caused by type 80/81 indicates that other properties as well, *e.g.* ability to develop resistance to more antibiotics than penicillin and streptomycin, may be necessary if the superiority over other phage types is to be maintained.

Relatively few members of phage group II were mercury resistant. This agrees with results published by *Richmond et al.* (1964) who demonstrated a correlation between high penicillinase production, never found with group II staphylococci, and resistance to mercuric chloride.

If strains with the same antibiogram were compared the occurrence of mercury resistant strains was found to be more frequent among TW — than among TW + strains. The reason why, as well as the reason for the positive correlation between antibiotic resistance and Tween negativity is still obscure. Tween negativity is known to be phage

TABLE 11

Mercury Resistance Correlated with Lipase Production among Group III Strains

Resistance to antibiotics	Percentage of mercury resistant strains			
	TW— = P_1	TW+ = P	$P_1 - P$	3 × sD
Sensitive	5.7 (331)	7.3 (1231)	1.6	4.6
P	15.6 (1227)	7.9 (3653)	7.7	3.3
PS	65.3 (138)	41.3 (201)	24.0	10.0
PST	89.0 (356)	73.7 ([illegible]81)	15.3	9.4
PSTE	97.0 (196)	69.2 (26)	27.8	27.5

Total number of strains is given in brackets
sD = the standard error of the difference
P = penicillin S = streptomycin T = tetracyclines E = erythromycin

The same results are obtained within the single but probably inhomogenous phage group III (Table 11) among the penicillin resistant (P PS PST) strains Among the TW— strains resistant to PSTE the percentage of mercury resistant is 97 whereas it is much lower (69) among the corresponding 26 TW + strains but there is no difference between the percentages in the two groups of sensitive strains

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In the previous report from 1963 (*Rosendal et al*) it was mentioned that strains resistant to tetracyclines and erythromycin were less numerous in the Danish material than among strains isolated in other countries

However if the present material is compared with other materials collected outside Denmark (*Jansson & Wager* 1963 *Fowler et al* 1963 *Frohman et al* 1964 *Bauer* 1965) it seems as if there is no such difference even though the materials concerned are smaller than the Danish ones and collected from more restricted areas In Denmark it was shown that the increase in the percentage of strains resistant to tetracyclines and erythromycin is primarily due to the spread of strains belonging to types 83A (*Rosendal & Jessen* 1964) and 6o57 (*Bulow* 1968c)

Fluctuations in the staphylococcal population similar to those found in the Danish material are also observed in other countries Increase in the percentage of multiple resistant strains is a common feature (*Bauer et al* 1960 *Krynski et al* 1966) from Australia (*Rountree et al* 1960) U S A (*Cohen et al* 1962) and Poland (*Krynski et al* 1966) it is reported that the incidences caused by strains of the 52 52A 80 81 complex are decreasing and that a multiple resistant epidemically occurring strain is introduced A strain with similar characteristics is described in reports from Canada (*Comtois & Bynoe* 1963) and the U K (*Temple & Blackburn* 1963) where according to *Jevons & Parker*

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dependent (*Rosendal et al* 1964) and the transduction experiments published by *Richmond & John* (1964) demonstrate a close linkage of penicillinase genes and those responsible for mercury resistance However future experimental work is needed in order to reveal the connexion between Tween negativity mercury resistance and multiple resistance to antibiotics

SUMMARY

During the years 1961–1965 66 219 strains isolated from 66 219 patients or staff members in Danish hospitals have been registered at Statens Seruminstitut

All the strains were phage typed and examined for production of a Tween 80 splitting enzyme 54 222 strains were examined for resistance to mercuric chloride and 58 419 strains for resistance to antibiotics

A steep decrease in the number of strains belonging to the 52 52A 80 81 complex was noticed whereas strains of phage types 83A and 6557 were isolated with increasing frequency

The percentage of strains isolated from cutaneous processes decreased considerably

Many more multiple resistant strains especially group III strains (including 83A and 6557) and non typable strains were isolated in 1965 than in 1962

A positive correlation between Tween negativity resistance to mercuric chloride and multiple antibiotic resistance was demonstrated among TW— strains the percentage of mercury resistant strains was found higher than among TW+ strains even when strains of identical resistance pattern were compared

Type 80/81 was more often mercury resistant than other phage types/groups were

The probability of the appearance of a new phage type resistant to methicillin is discussed

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THE FORMATION OF TOXOPLASMA ANTIBODIES IN RABBITS

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In a previous communication (19) the occurrence of an antibody like toxoplasma hostile factor in normal serum of several species was reported The results concerning the factor occurring in normal rabbit serum suggested that it was natural antibody to toxoplasma possibly having the nature of IgA It was proposed that this type of serum antibody may result from stimulation by toxoplasma antigen via the intestinal tract and not necessarily result from toxoplasma infection The present article which gives further support to this hypothesis describes the antibody response in rabbits following oral administration of non infectious toxoplasma antigen compared with the antibody formation following acute toxoplasma infection

MATERIALS AND METHODS

Animals Rabbits weighing between 2 5 and 3 0 kg and obtained from two different animal farms were used in the experiments They were housed in individual cages and fed carrots turnips and pellets containing cereal fish protein grass yeast, soy bean meal and vitamins The rabbits were b ed and tested for occurrence of anti toxoplasma factors in serum No seronegative rabbits as determined with the modified dye test (MA test) described below could be obtained at the time when the experiments were initiated The animals were g ouped according to the pre immun ization level in serum of antitoxoplasma activity Twent rabbits with serum MA test titres of 4–16 and 5 rabbits with titres of 64 or mor vere used in the expe iments to be described The rabbit sera obtained at diff ent times during the immunization courses were tested fo antibod activity within 2 days after the bleedings and later simultaneously with sera obtained at other bleed ng The ra we e stored t +4 C. A minor decrease in antibod activity v th t me w s observed f r some s ra but only after storage for more than a month

Toxoplasma antigen The peritoneal xudate of Sw s albino m e or cotton rats which had been infe ted three da s e rlier with li ng toxoplasma parasites was collected The exudate contained 5–10 m llion tox plasma parasites per ml The exudate was kept frozen for several v eeks then thawd and heated at 56 C for one hour To control that no infect us parasites were present in the heated antigen 0 5 ml was inje ted intraperitoneally nto f week-old m ce 6 for each batch of exudate The mice developed no signs of toxoplasma nfection

This study was supported by a PHS research grant AI 05074 05 from the *National Institute of Allergy and Infectious Diseases Publ c Health Service USA*

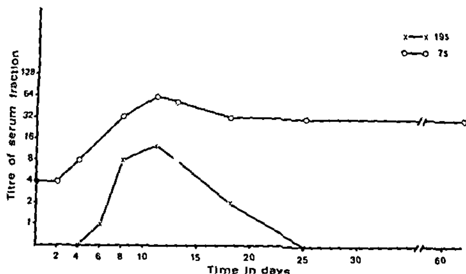


Fig 3

Antibody response in rabbits with pre existing serum 7S antibodies following inoculation with living toxoplasma parasites

Antibody activity was measured with the MA test Antibody titres in 7S and 19S serum fractions obtained by gel filtration of serum pools are indicated

An increase in antibody titre was already observed two days after the inoculation in two rabbits After four days an obvious increase in serum titre was observed in all rabbits Two rabbits died after 7 days and two after 9 days Pools were made of equal parts of the drawn sera and each pool was thereafter filtered on Sephadex G 200 Five ml fractions were collected and the antitoxoplasma activities of the fractions were determined by MA tests The newly formed antibodies were eluted with peak activities in either the first or the second protein peak of the chromatogram No maxima of antibody activity were observed between the first and second protein peaks in any serum during the whole time when the serum antibody titres were followed

Both 19S and 7S antibodies were detectable in unconcentrated fractions of the serum pool obtained four days after the inoculation of the parasites (Fig 2) The peak titre of 19S antibody was reached after 7–8 days whereafter there was a rapid fall in titre The 19S antibody persisted however in low titres for at least 4 months in one rabbit which survived following inoculation The possibility that this low antibody titre should be due to 7S contamination was contradicted by the fact that peak antibody activities also in late sera appeared in both the first and second protein peaks whereas no activity was eluted in the region between these peaks

The 7S antibody response had a biphasic appearance reaching one peak after ten days and then after a slow increase in titre a stable level after one month

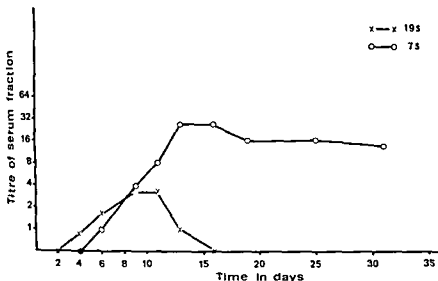


Fig 4

Antibody response in rabbits after immunization with killed toxoplasma parasites Antibody activity was measured with the MA test Antibody titres in 7S and 19S serum fractions obtained by gel filtration of serum pools are indicated

Antibody Response in Rabbits with Pre Existing 7S Toxoplasma Serum Antibodies Following Inoculation with Living Toxoplasma Parasites

Five rabbits with serum MA test antibody titres of 64 or more were selected for this experiment The antibody activity in a pool of these sera appeared in the second protein peak at Sephadex gel filtration and moved as slow γ globulins in Pevikon block electrophoresis No antibody activity was recovered in the first protein peak as judged by MA tests on concentrated fractions The rabbits were inoculated intravenously with 20 000 living toxoplasma parasites and the antibody response was followed as in the aforementioned experiment One rabbit died after 6 days and one after 18 days but three animals survived the infection

The rabbits responded to the infection by forming 7S as well as 19S antibodies (Fig 3) The lag phase before any demonstrable increase in antibodies appeared was 4 days for 7S antibodies and 6 days for 19S antibodies Peak titres for both antibody types were observed about 12 days after the inoculation The 19S response was transient and did not reach very high titre values The 7S antibody titre remained stable for at least 2 months after the peak titre had been reached

Antibody Response in Rabbits Inoculated Subcutaneously with Non Infectious Toxoplasma Antigen

Five rabbits with serum MA test antibody titres of 4 or 8 were injected subcutaneously once with 3 ml of undiluted heat treated toxo

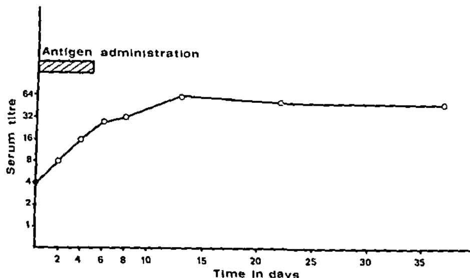


Fig 5

Antibody response in a rabbit following oral administration of non infectious toxoplasma antigen

Serum antibody titres measured with the MA test are indicated Antigen administration was continued for 5 days

plasma containing mouse peritoneal exudate The antitoxoplasma activity in the pooled pre immunization sera of these rabbits was eluted with the ascending limb of the second protein peak in gel filtration and had β globulin mobility The antibody response in the rabbits was followed by testing Sephadex G 200 fractions of the pooled sera

As in the case of toxoplasma infection the rabbits responded by forming both 19S and 7S antibodies the former reaching maximum titres earlier than the latter (Fig 4) About two weeks following the injection 19S antibodies were no longer detectable At about that time the 7S antibodies reached peak titres and remained thereafter at an essentially stable level For all the gel filtered serum pools the peak antibody activities were eluted in either the first or the second protein peak and not with the ascending limb of the second protein peak in any serum

Antibody Response in Rabbits after Oral Administration of Non Infectious Toxoplasma Antigen

Ten rabbits with pre immunization MA test titres of 4 or 8 were employed The antitoxoplasma activity in the pooled pre immunization sera was eluted slightly ahead of the second peak in gel filtration and moved as a β globulin in block electrophoresis The rabbits were fed daily with mouse peritoneal exudate containing heat inactivated toxo plasma parasites in the drinking water Water was withdrawn from the

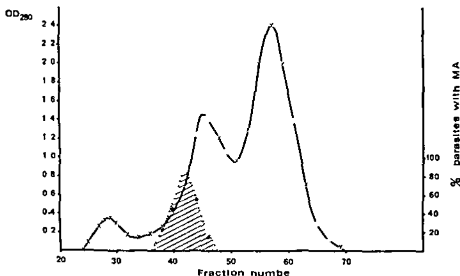


Fig 6

Gel filtration of rabbit serum obtained after oral administration of toxoplasma antigen The serum was obtained 15 days after the beginning of antigen administration Solid line indicates protein concentration measured as optical density (OD) at 280 mμ Shaded area represents toxoplasma antibody activity expressed as percentage of toxoplasma parasites showing morphological alterations (MA) after exposure to the serum fractions in the presence of activator

rabbits for some time before the administration of exudate and it was controlled that the whole amount of exudate was consumed by the animals Five rabbits were given 3 ml undiluted exudate per day and another five received 3 ml exudate diluted 1 5 with saline per day The administration of toxoplasma antigen was finished after 5 days

A significant increase in titre was observed in two of the five rabbits which were given undiluted exudate and in two of those which were given diluted exudate The antibody response in one of the rabbits is shown in Fig 5 Sephadex gel filtration of sera from this rabbit obtained before starting the immunization and at 6 15 and 28 days after that revealed that the antibodies in all cases were eluted somewhat ahead of the second protein peak (Fig 6) The results of block electrophoresis of these sera indicated that the antibodies moved as β globulins

The nature of the antibodies formed in response to oral administration of antigen was further studied by means of absorption with anti IgA and anti IgG sera For these experiments one part of the antiserum or antibody fraction to be tested diluted to contain 1–2 antibody units was mixed with one part of undiluted anti globulin serum heated at 56 C for 30 minutes After one hour at room temperature the mixtures were centrifuged and the supernatant obtained was tested for antitoxoplasma activity Sephadex G 200 fractions of rabbit immune sera containing either 7S or 19S antibodies and also diluted to contain 1–2 anti

body units were tested similarly as described above In all tests were included controls with Hanks solution substituting anti globulin sera and antitoxoplasma antibody solutions The antitoxoplasma antibodies in the sera of all the four rabbits which showed significant antibody rise in response to orally administered antigen could be absorbed by anti IgA serum but not by anti IgG serum (Table 1) Both the antibodies in serum 7S and 19S fractions could be absorbed to some extent by the anti IgA serum indicating a non specificity of this antiserum

TABLE 1

Absorption with Anti IgA and Anti IgG of 7S and 19S Fractions of Sera from Toxoplasma Infected Rabbits and Sera Obtained from Four Rabbits which had been fed Toxoplasma Antigen

	Samples						
				Antisera obtained after oral administration of antigen			
Absorption	Hanks solution	7S fraction	19S fraction	*Rabbit 1*	Rabbit 2	Rabbit 3	Rabbit 4
None	13	94	93	60	55	67	90
Anti IgA	14	89	68	31	22	39	30
Anti IgG	14	11	52	65	51	69	93

Figures refer to antitoxoplasma activity and indicate percentage of toxoplasma parasites showing morphological alterations after exposure to the various solutions in the presence of activator serum

DISCUSSION

The antibody response in rabbits to protein antigens has been claimed to consist of an initial 19S response followed by a 7S response (1 18) In case of stimulation with low doses of antigen only 19S response has been elicited (24) Recent studies have however indicated that 7S and 19S antibodies are synthesized simultaneously in the rabbit during the primary antibody response (2 8 17) The earlier studies were considered to have suffered because of the use of methods which detect 19S antibodies more readily than 7S antibodies In the experiments of the present report 19S antibodies appeared earlier in demonstrable quantitaties than 7S antibodies in some cases but in several experiments there was a simultaneous appearance in serum of the two antibody types Since 19S antibody appears more efficient in the MA test than 7S antibody (20) the present results are consistent with the consideration that 7S and 19S antibodies were synthesized concurrently in the rabbits irrespective of the pre existing serum antibody level

In the present experiments toxoplasma infection evoked both a 7S and 19S response irrespective of the pre immunization antibody levels

The antibody titres observed were however lower in the animals with high pre immunization antibody levels This could be due to an antibody suppressed formation of 19S antibody as well as 7S antibody (7 16) The enduring 19S response found in one rabbit is similar to what has been reported in humans with toxoplasmosis (13) The reason for this may be the continued occurrence of living parasites in the tissues after infection The production of 19S antibody is considered to require con tinued antigenic stimulation (22 25)

The antibodies formed in rabbits after antigenic stimulation have been shown to be heterogeneous with regard to electrophoretic and sedimentation properties (23) Antibodies with β globulin mobility and sedimentation coefficient between 7S and 19S have been observed after parenteral injection of a variety of antigens (11 14 23 27 28) This antibody type has been designated IgA (12) or IgA like (27) In the present experiments this antibody type was not noted after parenteral stimulation with toxoplasma antigen but since the methods used do not easily permit the distinguishing of such an antibody from 7S and 19S antibody it may very well have occurred After stimulation of rabbits with toxoplasma antigen by the oral route such antibodies did however appear exclusively These antibodies which also occurred in pre immun ization sera corresponded exactly to those described in an earlier paper as the toxoplasma hostile factor (19) The reasons for characterizing these antibodies as IgA like were their electrophoretic mobility be haviour on gel filtration heat lability and sensitivity to 2 mercapto ethanol Although no specific anti heavy chain sera were employed in the present study the results of absorption experiments indicated that the antibodies formed after oral administration of antigen were not IgG and presumably not IgM The possibility that they should be IgM was contradicted by their behaviour on gel filtration There was thus an accumulated evidence that the antibodies formed after oral administra tion of antigen were IgA like

The same type of antibody as that normally occurring in the majority of tested rabbit sera (19) could be evoked by stimulation of rabbits with toxoplasma antigens via the oral route This observation strongly indicates that the toxoplasma hostile factor occurring in animal sera may be the result of a similar antigenic stimulation and not necessarily result from toxoplasma infection The observation that other antibody types were formed when other routes of antigen administra tion were employed stresses the importance of the oral route for the formation of the IgA like toxoplasma antibody

The demonstration of an immune response after oral administration of antigen is in agreement with previous studies (3 6 14) The IgA like serum antibodies may possibly originate from a local antibody pro duction in the intestinal mucosa as the immunoglobulin producing cells in this location of the rabbit contain mainly IgA (5)

The recent findings of *Rothberg et al* (14) are at variance with th

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KINETICS OF THE *IN VITRO* IMMUNOINACTIVATION OF *TOXOPLASMA GONDII*

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In the presence of components of normal human serum the reaction of specific antibodies with toxoplasma parasites will lead to destruction and death of the parasites The morphological alterations caused by this immunoinactivation can be observed after staining the parasites with methylene blue as in the Sabin Feldman dye test (16) or by phase contrast microscopy (6) The kinetics of the inactivation of toxoplasma by antibody and human serum (activator) have been briefly dealt with by *van Soestbergen* (17) and *Lycke et al* (10) In the present report the immunoinactivation of toxoplasma is studied and discussed in relation to the complement dependent systems immune haemolysis haemolysis of paroxysmal nocturnal haemoglobinuria (PNH) cells and bacteriolysis

MATERIALS AND METHODS

Parasites The RH strain of *Toxoplasma gondii* was used The parasites were obtained from the peritoneal exudate of toxoplasma infected Swiss albino mice Parasite suspensions containing 5×10^6 to 10 parasites per ml of Hanks balanced salt solution and prepared as described previously (9) were used

Activator sera *Human sera obtained from apparently healthy blood donors and* devoid of demonstrable toxoplasma antibody activity as measured with the modified dye test described below were used as activator sera The sera were stored deep frozen until used

Immune sera All immune sera were treated at 56 C for 30 minutes before use in the experiments to be described One human immune serum (Sk) with a dye test titre of 8000 also used in previous experiments (18 19) was employed As judged by Sephadex G 200 gel filtration the toxoplasma antibodies in this serum were mainly of the 7S variety but a minor 19S antibody activity was also demonstrable This serum was used as a reference and if not otherwise stated it was used as the source of antibody in the experiments to be described

A 19S antibody fraction was obtained from the serum (SH) of a patient who had noticed lymphadenopathy for about two weeks This serum had a dye test titre of 250 All detectable toxoplasma antibody activity after gel filtration on Sephadex G 200 was located in the 19S peak

In some experiments a rabbit immune serum (R 16) or fractions of this serum obtained by gel filtration were used This serum was obtained 8 days after intra

This study was supported by PHS research grant AI 05074 04 from the *National Institute of Allergy and Infectious Diseases Public Health Service USA* The excellent technical assistance of Miss *Mona Eriksson* is gratefully acknowledged

venous inoculation of about 50 000 toxoplasma parasites and contained 7S as well as 19S antibodies

The amount of antibody capable of giving morphological alterations (MA) in 50 per cent of the exposed toxoplasma parasites after one hour's incubation in the presence of activator serum is in the following referred to as one antibody unit

Kinetic studies In the following the term immunoinactivation is used to describe the induction of MA of toxoplasma parasites although the loss of the various functions of the parasites does not always parallel the development of MA (20) The method used for determination of toxoplasma antibody activity was a modified dye test (18) previously referred to as a test on morphological alterations As a diluent for all reactants in the tests was used Hanks balanced salt solution containing Mg and Ca^{++} In the experiments to be described the reactants consisted of one part antibody solution one part parasite suspension and two parts of activator serum if not otherwise stated The reactants were separately put in an incubator and brought to the desired reaction temperature before performing the tests All glassware used in the experiments was also temperature stabilized before use

After some minutes aliquots of the reactants were put in a glass tube and thoroughly mixed with the aid of a pipette Immediately thereafter drops of the mixture were placed on slides under coverslips At varying intervals slides were removed from the incubator and the percentage of parasites with MA was determined

Gel filtration Gel filtration on Sephadex G 200 was performed as described previously (19) Fractions each with a volume of 5 ml were collected and the unconcentrated top fractions of the first and second peaks of the eluate were used as sources of 19S and 7S antibodies respectively

RESULTS

Immunoinactivation of Toxoplasma at Different Concentrations of Antibody and Activator Serum

In a series of experiments one part of toxoplasma parasite suspension (5×10 -10 parasites per ml) was mixed with two parts of activator serum and one part of a solution of antibody in varying concentrations The antibodies used were obtained from the human immune serum (Sk) with toxoplasma antibodies mainly of the 7S variety

Immediately before mixing the reactants and then after various

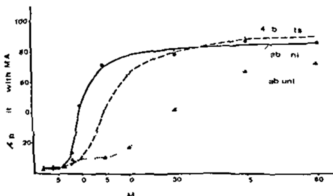


Fig 1

Kinetics of toxoplasma immunoinactivation at various antibody concentrations
Human serum (Sk) was used as antibody source
The antibody (ab) concentrations tested were 1 4 and 16 units.

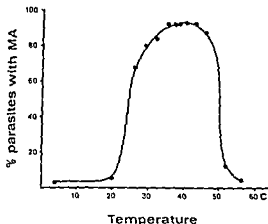


Fig 5

Influence of temperature on toxoplasma immunoinactivation
Percentages of parasites with MA observed after incubation for one hour at vari
temperatures (C) are plotted Four antibody units of human serum (Sh) were us

a waterbath for one hour at different temperatures The optimal tem perature for the immunoinactivation was in all experiments found to be within the range of 35–43 C (Fig 5)

The progress of the immunoinactivation of toxoplasma at 27 C compared to that at 37 C is shown in Fig 6 In this experiment 8 antibody units of the human immune reference serum Sh were used The In_{50} period was considerably longer at 27 C than at 37° C and the rate of the reaction was decreased The temperature coefficient (Q_{10}) calculated as the quotient between maximal reaction rates at 37 C and 27° C was found to have a value of approximately 10

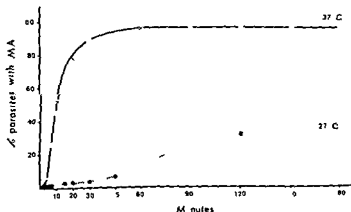


Fig 6

Kinetics of toxoplasma immunoinactivation at 27 C and 37 C
Eight antibody units of human serum (Sh) were used.

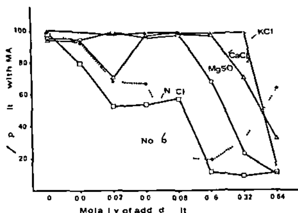


Fig 7

Effect of salt concentration on toxoplasma immunoinactivation

Molarities indicated on the abscissa are final molarities of added salt solution With no salt added the molarity was 0 053 due to presence of serum In the experiment where serum was omitted the basal molarity was adjusted to 0 053 by adding NaCl The symbols indicate results obtained with One antibody unit and NaCl (squares) Four antibody units and $MgSO_4$ (circles) Four antibody units and KCl (crosses) Forty antibody units and CaCl (triangles) Filled circles connected by dotted line indicate results obtained with NaCl in the absence of antibody and activator

Influence of Salt Concentration on the Immunoinactivation of Toxoplasma

One part (0 3 ml) of human immune serum diluted 1 100 1 1000 or 1 4000 with 0 0075 M saline was mixed with one part activator serum and one part of either distilled water or salt solution The molarities of the salt solutions ranged from 0 03 to 1 92 Estimating the molarity of whole human serum at 0 15 the lowest molarity of the resulting mixtures was thus 0 053 and the highest 0 693 The various mixtures were thereafter each added to about 3 million sedimented toxoplasma parasites from which the supernatant had been carefully removed After incubation for one hour at 37 C the parasites in each mixture were counted in a haemocytometer and the percentage of parasites with MA was determined Representative results are compiled in Fig 7 The molarities indicated in the figure refer to added salt and do not include the basal molarity of 0 053

At high salt concentrations the development of MA of toxoplasma was inhibited At the same antibody and salt concentrations the salts with high ionic strength (CaCl $MgSO_4$) were more inhibitory than salts with low ionic strength (KCl NaCl) No difference in inhibiting capacity between NaCl and KCl was observed By increasing the antibody concentration the inhibition at high salt concentration could be counteracted With all salts tested maximal inactivation of toxoplasma was encountered at molarities in the range of 0 1–0 2 In several experiments less effective inactivation was observed at a molarity of about 0 07

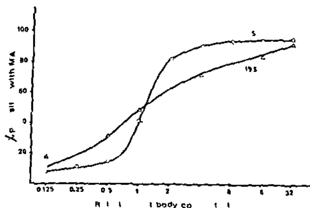


Fig 10

Inactivation of toxoplasma by various concentrations of 7S and 19S antibodies
The results of inactivation of toxoplasma by varying amounts of 7S or 19S antibodies in the presence of activator serum are plotted The 7S and 19S antibodies were obtained from different Sephadex C 200 fractions of an early rabbit immune serum (R 16)

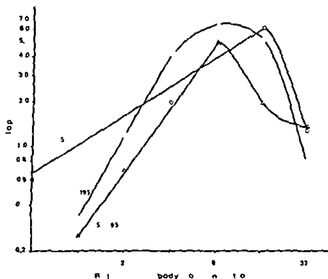


Fig 11

Reaction rate of toxoplasma inactivation at various concentrations of 7S and 19S antibodies The 7S and 19S antibodies were obtained from different Sephadex C 200 fractions of a rabbit serum (R 16) Reaction rates are plotted as maximum slopes of the kinetic inactivation curves obtained at different antibody concentrations Results obtained with 7S or 19S antibodies and mixtures of equal parts of 7S and 19S antibodies are given

to 10 minutes Within the range of 1–8 antibody units the lag phase was 2–4 minutes shorter for 19S than for 7S antibodies when antibody solutions with identical titres were used The inactivation curves obtained with 7S and 19S antibodies showed that the reaction rate was dependent on antibody concentration When the maximal reaction rates

indicated as slopes at the inflection point of the sigmoidal curves were plotted against antibody concentration the curves shown in Fig 11 were obtained The 7S as well as 19S antibody dose response relationships were linear indicating that up to certain limits the reaction rate was directly correlated to antibody concentration At high antibody concentration the reaction rate was decreased This was observed at a lower relative antibody concentration measured as antibody units for 19S antibodies than for 7S antibodies Although a decreased reaction rate was regularly observed at high concentrations of antibodies corresponding zone phenomena were very often not observed at test readings after incubation for one hour (cf Figs 10 and 11) Thus the final results of the tests were often equal although the reaction rates were distinctly different

DISCUSSION

The kinetics of the immunoinactivation *in vitro* of toxoplasma parasites by antibodies and activator factors (the properdin system) may be described by a curve with a sigmoidal shape This suggests that the immunoinactivation is complex requiring a sequence of several steps Curves of a sigmoidal shape are also found in immune haemolysis (11) and bactericidal reactions (15)

The length of the initial lag period in the immunoinactivation of toxoplasma was inversely correlated to the amount of antibody and activator components participating in the reaction The rate of the inactivation was dependent on both the concentration of antibody and activator factors in the reaction mixture Thus the immunoinactivation of toxoplasma seems to be a reaction of high order the rate of which is dependent upon the product of the concentration of two or more reactants There was also observed an obvious influence on the reaction rate by varying the number of parasites in the tests This observation differs from what has been reported for bactericidal reactions which have been claimed to be first order reactions the rates of which are not affected by the concentration of test organisms (3) A Q_{10} of about 10 observed in the present experiments indicates a high energy of activation compared to that needed for bactericidal reactions (3) This again might reflect the complexity of the immunoinactivation of toxoplasma

The immunoinactivation of toxoplasma seems to have more narrow temperature requirements than immune haemolysis (7) and bacteriolysis (22) *Leon* has shown that the optimal temperature for lysis of sheep red cells using human complement is 30–32 C but that an appreciable lysis is also obtained at 22 C and 27 C (7)

The finding of an inhibiting effect of high salt concentrations for the development of MA agrees with what has earlier been found by *van Soestbergen* (17) This author did not however test his system at molarities lower than 0 271 The results of the present experiments indicated

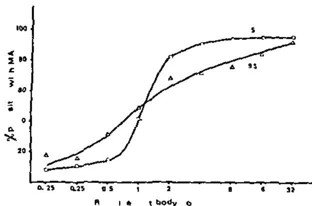


Fig 10

Inactivation of toxoplasma by various concentrations of 7S and 19S antibodies The results of inactivation of toxoplasma by varying amounts of 7S or 19S antibod in the presence of activator serum are plotted The 7S and 19S antibodies w obtained from different Sephadex G 200 fractions of an early rabbit immune seri (R 16)

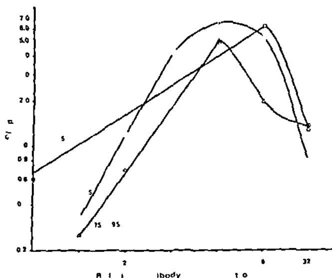


Fig 11

Reaction rate of toxoplasma inactivation at various concentrations of 7S and 19S antibodies The 7S and 19S antibodies were obtained from different Sephadex G 200 fractions of a rabbit serum (R 16) Reaction rates are plotted as maximum slopes of the kinetic inactivation curves obtained at different antibody concentrations Results obtained with 7S or 19S antibodies and mixtures of equal parts of 7S and 19S antibodies are given

to 10 minutes Within the range of 1–8 antibody units the lag phase was 2–4 minutes shorter for 19S than for 7S antibodies when antibody solutions with identical titres were used The inactivation curves ob tained with 7S and 19S antibodies showed that the reaction rate was dependent on antibody concentration When the maximal reaction rates

for 19S antibodies. This finding agrees with those obtained for mouse haemolytic and cytotoxic antibodies (13) and indicates that fewer 19S than 7S antibody molecules are needed for parasite inactivation. The results of *Robbins et al.* (14) indicated that IgM was 120 times as potent as the IgG in sensitizing bacteria for complement dependent killing. Borsos & Rapp (2) have recently proposed that on cell surfaces a single molecule of 19S antibody suffices to fix complement while doublets are required for 7S antibodies.

The linear relationship observed between velocity of toxoplasma immunoinactivation and concentration of antibody is similar to the results reported by *Mayer et al.* for immune haemolysis (11). In the latter investigations no reduction in reaction rate was however observed at antibody excess. The reduction of reaction rates with 19S and to a less degree 7S antibody excess observed in the present study is similar to the wellknown prozone phenomenon. The explanation for this could be that when present in excess the larger 19S antibody molecules rather than the smaller 7S molecules are liable to cover receptors for complement components and thus interfere with the production of damage in the parasite cell membrane. Prozone inhibition of bactericidal activity has recently been reported for both IgG and IgM antibodies (14).

SUMMARY

The kinetics of the immunoinactivation of *Toxoplasma gondii* by corresponding antibodies and human activator serum were studied. The induction of morphological alterations of the toxoplasma parasites followed a sigmoidal course. The influence of temperature, pH and ionic strength for the immunoinactivation of toxoplasma was studied and discussed in relation to requirements for various complement dependent systems.

The effects of 7S and 19S antibodies on toxoplasma were compared. Both types of antibody required activator factors. The relative efficiency per molecule seemed to be greater for 19S than for 7S antibodies. The toxoplasma inactivation rate was inhibited to a greater degree by high concentrations of 19S antibodies than by 7S antibodies at corresponding titres.

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The Municipal Virological Laboratory and the Department of Virology the Institute of Microbiology University of Gothenburg Sweden

AN ELECTRON MICROSCOPIC STUDY ON THE IMMUNOINACTIVATION OF *TOXOPLASMA GONDII*

By

ÖRJAN STRANNEGÅRD

Received 11 vii 67

The immunoinactivation of *Toxoplasma gondii* requires in addition to antibody activator factors present in normal human serum The mode of action of these factors considered to be identical with the factors of the so called properdin system (5 7) has recently been studied in our laboratory using a cell culture method and a modified dye test to demonstrate the immunoinactivation (12)

With the aid of electron microscopy employing ferritin labelled antibody the attachment of antibodies to toxoplasma and to the membrane surrounding intracellular toxoplasma parasites has been studied (10 13) In the present study electron microscopy was used to elucidate the interdependent actions of antibody and the activator factors for the immunoinactivation of toxoplasma

MATERIALS AND METHODS

Activator sera Human sera devoid of detectable antitoxoplasma activity as measured with the modified dye test described below and obtained from apparently healthy blood donors were used as activator se a The sera were stored deep f ozen until used

Immune sera For ferritin-conjugation of 7S and 19S antibodies the serum of a rabbit which had been nfected 8 days earlier with about 50 000 li ing toxoplasma parasites was selected The serum was filtered through a 90 cm × 3 cm column of Sephadex G 200 gel using 0 15 M phosfate buffer pH 7 4 for the elution The fractions comprising the first an l econd protein peaks in the chromatogram we collected and concentrated 5 f ld by dial sis against Carb x No contamination of 7S immunoglobulin in the concentrated 19S fraction or ice versa w s demonstrable using sucrose gradient ultracentrifugation (9) The f actions vere subsequently conjugated with fe tin The dye test titre after conjugation w s 256 for the 19S fraction and 128 for the 7S fraction

In some experim nts a rabbit serum obtained 3 months ft r inoculation with 50 000 toxoplasm pa sites was used Gel filtration h wed that lmost all anti toxoplasma activit of the serum was located in th 7S f t ons The serum was precipitated with h lf saturated ammonium sulf te and then conjugated with ferritin The dye t t titre of the conjugate was ? (

Properdin In the following the term properdin refers to a preparation of p o

This stu ly wa up ported by a PHS research gr nt AI 05074 05 from the *Nat onal Institute of All rg an l Infecti us Diseases I ubl c Health Service USA* The author wishes to ackn ledge the very skilful technical assistance of Miss *Ba bro Anders n*

perdin obtained from AB KABI Sweden and described in earlier papers (19 21) The preparation contained 16 000 units properdin per gram as measured with the zymosan assay (16) There was no demonstrable lysozyme in the preparation as determined with the method using Micrococcus lysodeikticus described by *Parry et al* (15) *The term properdin is used although it cannot be stated that the factor active in the zymosan assay is identical with the one taking part in the immunoinactivation of toxoplasma*

Toxoplasma parasites The RH strain of *Toxoplasma gondii* was used The parasites were obtained from the peritoneal exudate of toxoplasma infected Swiss albino mice In the experiments to be described the parasite suspensions used were washed and freed from most of the exudate cells and cellular debris by differential centrifugation as described by *Lycke & Lund* (11) In some experiments with the aim of studying intracellular parasites untreated exudate was used

Test on morphological alterations of toxoplasma parasites The test principally a modification of the Sabin Feldman dye test has been described previously (21)

Coupling of ferritin to antibody The method of *Singer & Schick* (17) using toluene 2 4-diisocyanate was followed The ferritin used was 2 × crystallized of horse spleen origin and obtained from Nutritional Biochemicals Corporation Cleveland Ohio USA After conjugation parts of the globulin solutions were centrifuged at 35 000 r p m for 2 hours in a Spinco model 1 ultracentrifuge using a SW 39 rotor in order to remove unconjugated antibody (17)

Experimental procedure Washed toxoplasma parasites were suspended in Hanks solution at a concentration of 10 million parasites per ml One ml aliquots of the parasite suspension were then mixed with antibody activator serum and properdin or combinations of these three varying the concentrations of the reactants The final volume of each mixture was 1 5 ml In some experiments the parasites were pre exposed to one of the reactants of the test for 15 min at 37 C before mixing with the other reactants After mixing all samples were incubated at 37 C for one hour The parasites were then pelleted by centrifugation at 440 g for 10 min at room temperature The pellets were fixed in 1 per cent osmium tetroxide made according to *Palade* (14) for 2 hours at 4 C and thereafter dehydrated by passage through a graded series of ethyl alcohol solutions The pellets were finally embedded in Epon and sectioned No special staining was used The sections were examined in a Siemens Elmiskop I or a JEM T7 electron microscope and the majority of plates taken at 19 000 diameters magnification

RESULTS

Attachment of Toxoplasma Antibody to the Parasite Surface

After exposure of toxoplasma parasites to ferritin labelled antibody of the 7S as well as 19S type ferritin particles were found lining the surface of the parasites (Figs 1 and 2) The appearance of ferritin particles on the parasite surface could be almost completely inhibited by pre treating the parasites with unconjugated 7S or 19S antibody before adding ferritin labelled antibody

Parasites exposed to conjugated 19S antibody exhibited much fewer ferritin particles than parasites exposed to 7S antibody although the

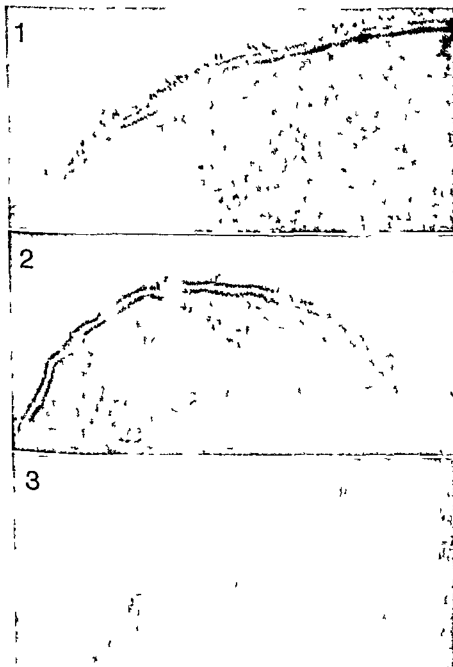
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Figs 1–3

Fig 1 Toxoplasma parasite incubated with ferritin labelled 7S antibody Magnification × 120 000

Fig 2 Toxoplasma parasite incubated with ferritin labelled 19S antibody Magnification × 120 000

Fig 3 Toxoplasma parasite incubated with ferritin labelled 7S antibody and activator Only a few ferritin particles are seen on the surface of the parasite even in places where the double membrane appears intact Scattered ferritin particles appear in the interior of the parasite Magnification × 120 000


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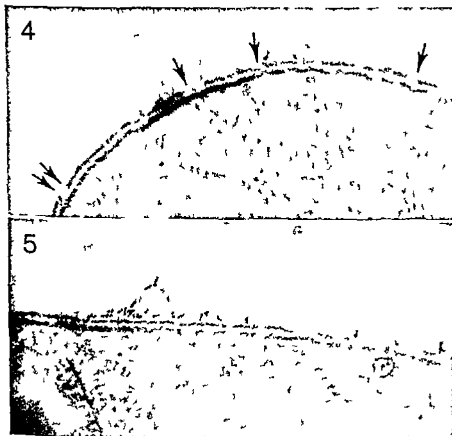
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Fig 3 Toxoplasma parasite incubated with ferritin labelled 7S antibody and activator Only a few ferritin particles are seen on the surface of the parasite even in places where the double membrane appears intact Scattered ferritin particles appear in the interior of the parasite Magnification × 120 000



Figs 4–5

Fig 4 Toxoplasma parasite incul ated with ferritin-conjugated 7S antibody an l properdin at a final concentration of 40 units per ml Note that only scarce ferritin particles are visible on the surface and the d stortion and holes in the outer membrane (arro s) Magnification × 1?0 000

Fig 5 Toxoplasma parasite incubated with ferritin conjugated /S antib ly activa tor and properdin used in a final concentr tion of 50 un ts ; e ml Note scarcity of ferritin pa t cles on the surface and holes in the out membr ne but intact inner membrane Magnification × 1?0 000

particles compared to par isites treated with antibody and activator only Although the membranes appeared in general much less damaged in the presence of high conc ntrations of properdin evident holes in the outer membrane could n(rtheless in some c ises b l monstrated (Fig 5) No penetration o f ritin particles through the pellicle was however observed

DISCUSSION

The present results agree well with earlier investigations by others in regard to the attachment of ferritin labelled antibody to the toxoplasma

parasite surface (10 13) and the binding of antibody to the membrane in infected cells limiting the toxoplasma containing vacuolar space (13) The latter finding strongly suggests that the limiting membrane is produced by toxoplasma The parasite membrane appears to act as an effective barrier to the ferritin labelled antibody in the absence of activator factors A similar observation was made by *Goldberg & Green* (6) and by *Easton et al* who studied the action of ferritin labelled antibody on ascites tumour cells (4)

When acted upon by antibody toxoplasma parasites retain their capacity to penetrate their host cells (12) and to consume oxygen (22) provided activator factors are not present Antibody alone seems thus not to impair the parasites noticeably These findings get support from the present investigation since no effect of antibody alone on the cell membrane or inner structures of the parasites could be demonstrated It should be mentioned however that for ascites tumour cells *Dumonde et al* (3) have shown that immune reactions confined to the cell surface may be able to induce changes in the cytoplasm without acutely impairing the viability of the cell

In the presence of activator serum antibody caused extensive damage to the parasite pellicle allowing antibody to penetrate through the membranes The ferritin particles disappeared almost completely from the surface of the parasites also in places where the membranes appeared relatively intact The latter finding agrees with what has been reported by *Ludvik et al* (10) Such a disappearence of antibody from the cell surface does not seem to occur at least not to the same extent in immune cytolysis (4)

The action of antibody and activator resulted after the disruption of the cell membrane in an evident loss of substance of the parasite The latter observation is consistent with earlier electron microscopic investigations (2) and also with the results of histochemical studies (8) showing that ribonucleic acid disappears from the parasites following the exposure to antibody and activator The positive dye test is thus probably the result primarily of a damage to the parasite membrane followed by colloid osmosis and not as has been claimed by *Braunsteiner et al* (1) the result of destruction of the inner structures of the parasites by specific antibody The loss of inner structure possibly involving lysosomal activation should then be regarded as a secondary phenomenon

Although ferritin labelled antibody solutions giving about the same antibody titres were used for 7S and 19S antibodies the amount of ferritin particles attached to the parasite surfaces was much less when 19S antibodies were employed than when 7S antibodies were used This finding is consistent with the observation (20) that 19S antibody is more efficient per molecule than 7S antibody in the immunoinactivation of toxoplasma The possibility remains however that ferritin is less readily conjugated with 19S than with 7S antibody

Previous studies (21) indicated that a serum constituent called properdin as it was prepared according to a procedure used for obtaining this substance (18) and also highly reactive in the zymosan assay of *Pillemer et al* (16) was necessary for the immunoinactivation of toxoplasma The results suggested that properdin in the presence of antibody was capable of injuring the toxoplasma parasite and in addition it was proposed that properdin might be able to dissociate antibody from the toxoplasma antigens (21) These postulated effects of properdin get further support from the present investigation The disappearance of antibody from the surface observed when activator serum was added to toxoplasma parasites sensitized with antibody was also seen when the properdin preparation instead of whole activator serum was added to the parasites Although there was no complete disappearance of antibody from the parasite surface the release of antibody observed may have been enough to inhibit the immunoinactivation of toxoplasma In accord with this assumption there did not occur any complete disruption of the parasite cell wall with entrance of antibody into the parasites when the toxoplasma parasites were treated with antibody activator and a high concentration of the properdin preparation The finding of an inhibiting effect of high concentrations of properdin is in accord with the results of *Wardlaw & Pillemer* who observed that an excess of properdin inhibited the bactericidal effect of normal human serum on *Shigella dysenteriae* (23)

The effect of the properdin preparation and antibody in the absence of other added activator factors was recognized as a slight derangement of the parasite cell membrane This finding agrees with the observation made with phase contrast microscopy that toxoplasma parasites exposed to the properdin preparation and antibody exhibited morphological alterations (21) Although the properdin preparation employed was devoid of demonstrable complement components measured with R reagents (21) it cannot be excluded however that it contained complement in sufficient amounts to cause the observed derangement of the parasite membrane

SUMMARY

The immunoinactivation of toxoplasma was studied electron microscopically with the aid of ferritin labeled antibody of the 7S as well as 19S type In the absence of activator serum antibody was distributed on the surface of the parasites without causing any evident structural damage In the presence of activator serum the parasite membrane was extensively damaged the antibodies disappeared from the parasite surface and were observed in the interior of the parasites The 19S antibody type appeared more effective per molecule than 7S antibody With a preparation of properdin added to antibody minor effects on the parasite cell membrane were observed Properdin seemed capable of dissociating antibody from surface antigens

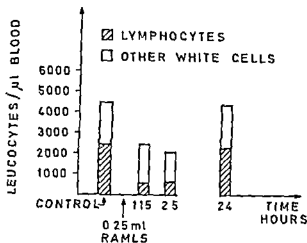


Fig 1

Course of total white blood cell counts and lymphocytes in five mice 17 hours before and 70 and 150 minutes and 24 hours after intraperitoneal injection of 0 25 ml RAMLS

g A single cell suspension was prepared by pressing the excised organs through a stainless steel mesh into phosphate buffered saline (PBS) —Ca —Mg The cells were washed two to three times in the same fluid and counted in a standard haemocytometer with methyl violet in acetic acid and eosin the counts obtained in this way were always almost identical The cells were then resuspended in an appropriate volume of PBS and emulsified with an equal volume of complete Freund s adjuvant to give a final concentration of 100 or 200 × 10^6 lymphoid cells in 0 8 ml Rabbits received 0 2 ml of this suspension in each of the footpads Four weeks later booster injections of 100 × 10^6 spleen lymphoid cells in PBS without adjuvant were given intraperitoneally on three consecutive days and one week after the last injection the rabbits were bled from an ear vein The blood was allowed to clot at room temperature and then kept overnight at 4 C The serum was separated pooled and then stored at −20 C after the addition of merthiolate 1 10 000 The rabbits have been bled several times and booster injections of 100 × 10 spleen lymphoid cells were always given one week prior to bleeding Serum has not been decomplemented except for *in vitro* experiments when it was heated to 56 C for 30 minutes Strictly speaking the serum prepared in this way is a rabbit antiserum to mouse lymph node and spleen cells but for the sake of simplicity it will be referred to as rabbit anti mouse lymphocytic serum (RAMLS)

Serum obtained from rabbits injected into each of the footpads with 0 2 ml mixture of equal volumes of complete Freund s adjuvant in PBS without cells (CFA serum) and normal rabbit serum (NRS) were used for controls These sera were treated and stored in exactly the same manner as the RAMLS

Effect of RAMLS on Total White Blood Cell and Lymphocyte Counts

Five normal C3H female mice two to three months old weighing approximately 20 g were used Total and differential counts of white blood cells in the blood were made after staining with methyl violet in acetic acid and May Grünwald Giemsa staining respectively On the following day 0 25 ml RAMLS was injected intraperitoneally into each of the mice and 70 and 150 minutes and 24 hours later blood samples were examined in the manner described above The results are shown in Fig 1 as mean values for the five mice There is a fall in the total count to approximately half of the original values within 150 minutes but already 24 hours later the levels are almost completely restored It must be emphasized that the control sera were not tested similarly

Cytotoxic Test

A slight modification of *Gorer & O Gorman*s technique (4) was used Equal volumes of a suspension of spleen lymphoid cells in PBS —Ca —Mg (10 and 50 × 10^6 cells/ml respectively) an 1 4 dilution of RAMLS or NRS which was decomplemented for 30 minutes at 56 C and fresh guinea pig serum was incubated for 30 minutes at 37 C. Then one drop of freshly prepared 0 25 per cent trypan blue solution was added and the number of living (unstained) and dead (stained) cells was determined The cytotoxic index (8) was calculated as

$$\frac{\text{per cent unstained in control—per cent unstained in test}}{\text{per cent unstained in control}}$$

where values >0 15 were considered positive Indices of 0 24 and 0 18 were obtained with the cell concentrations of 10 and 50 × 10^6/ml respectively

The LCM virus strain used has been described in an earlier report (20) with the amendment that for the present investigation it was passed in C3H mice

The virus titrations of blood samples were as previously described (21) The end points were calculated according to the method of *Reed & Muench* (17) and expressed as log 10 I D /0 03 ml

Complement Fixing Antibodies

The preparation of antigen and the standard technique used for the test have been described in a previous paper (24) this method was altered only in that the antigen is now produced from the spleens of infected mice The complement fixation tests were usually performed in acryl plates the smallest dilution used being 1 4 However a few tests were performed in glass tubes the smallest dilution here being 1 8 Since a proportion of the sera were anti-complementary some of the sera were incubated with guinea pig complement at 37 C for one hour and thereafter decomplemented at 56 C for 30 minutes The anticomplementary effect was thereby abolished

RESULTS

In a pilot experiment five normal adult female C3H mice were given 0 25 ml RAMLS intraperitoneally for seven consecutive days and on the third day after initiation of serum treatment the animals were infected with 1000 LD_{50} LCM virus intraperitoneally The virus titres in the blood rapidly rose to the virus carrier level (22) about $10^{3.5}$ and in the three surviving mice the titres have remained at this high level for more than 12 months Apart from an initial rather steep rise in complement fixing antibodies which disappeared in 17 days there was a small rise in these antibodies from the 45 h to the 60th day of the experiment Since then all three mice have had a complement fixing antibody titre of < 1 4 An identical group of mice in the pilot experiment received 1000 LD LCM virus intraperitoneally but no RAMLS During the first weeks these mice occasionally showed very low virus titres in the blood but since then no virus has been detected in the blood The antibody titres rose rapidly to levels about 1 128 and remained at this level

These results led us to the actual experimental design in which we used one experimental group A which received RAMLS + virus and three control groups B virus alone C CFA serum + virus and D NRS + virus The experimental group A consisted of ten normal C3H female mice about three months old weighing 20–25 g These mice

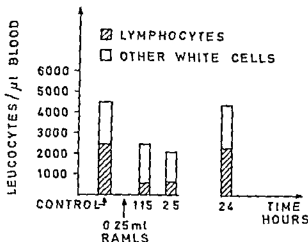


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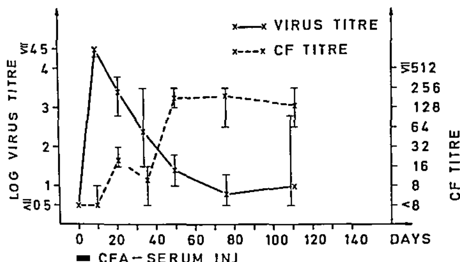


Fig 3

Course of virus titres and CF antibodies in five mice receiving 1000 LD$_{50}$ LCM virus day 0 and 0 25 ml CFA serum days —1 to +5

a single injection of 1000 LD$_{50}$ LCM virus intraperitoneally but no serum The mice were then observed at the same intervals as those in the experimental group A with virus titrations in the dilutions 10^{-1}–10^{-4} or 10^{-1}–10^{-3} and determination of complement fixing antibodies in serum The mean virus titre of these eight mice has remained ≦ 10$^{0.5}$ throughout the experiment a few titrations being above this level during the first three weeks A single mouse forms an exception in that it displayed moderately elevated virus titres on several occasions after the first three weeks The complement fixing antibodies in all the mice rose within three weeks to a level of about 1 128 and they have since remained at that level

The control group C consisted of five normal C3H female mice two to three months old weighing about 20 g These mice were treated for six consecutive days with 0 25 ml intraperitoneally of a serum made by injection into rabbits of complete Freund s adjuvant alone (CFA serum) On the day after initiation of the serum treatment they also received 1000 LD$_{50}$ LCM virus intraperitoneally The mice were then observed on days 10 21 35 50 76 and 111 after the virus inoculation with determination of blood virus titres and complement fixing antibodies in serum The blood samples were titrated on mice in the dilutions 10^{-1}–10^{-4} The titres rose steeply in all five mice on the tenth day being ≧ 10$^{4.5}$ During the following weeks the virus titres gradually declined and now approximated those found in group B (Fig 3) The complement fixing antibodies slowly rose to a level of about 1 128 One of the five mice died after 35 days in direct relation to collection of a blood sample without previous signs of illness

One control group D consisted of normal C3H mice, four females and three males, two to three months old, weighing about 20 g. These mice were given normal rabbit serum (NRS) 0.25 ml intraperitoneally for six consecutive days and in addition on the day after initiation of serum treatment 1000 LD_{50} LCM virus intraperitoneally. The mice were then observed on days 10, 21, 30, 45 and 59 with determination of blood virus titres in the dilutions 10^{1}–10^{-8} and complement fixing antibodies, the first dilution being 1:8. The virus titres rose rapidly as in group C and remained at a level of about $10^{3.3}$ for a month and then declined. Two months later no virus could be found in the blood of half of the mice and only very low virus titres in the rest. A single mouse reached a titre of only $10^{1.5}$ on day 10 and since then it has had no trace of virus in the blood. In the course of a month the complement fixing antibodies rose to levels of about 1:128 as in group C. One mouse died between day 21 and day 30 without previous signs of disease.

DISCUSSION AND CONCLUSION

The pilot experiment demonstrated that short term administration to immunologically mature mice of an anti-lymphocytic serum and simultaneously a single LCM virus inoculation would make the mice tolerant to the virus for at least one year. In the present experiment the tolerance has to date been present for seven months. In some of the mice complement fixing antibodies have never been demonstrated with certainty, whilst the remainder of the mice displayed these antibodies for a short period after which they seemed to disappear again. The course of virus titres and the absence of antibodies in the completely tolerant mice cannot be distinguished from these characteristics in mice which were inoculated with virus neonatally (*i.e.* under the age of 18 hours) (23). It is a little more difficult to predict the course in the remaining mice, but the results hitherto obtained seem to indicate that the tolerance will also prove to be lifelong in these mice.

An interesting feature is our observation of a small rise in complement fixing antibodies that had not the slightest influence on the viraemia. Similar observations were made in a previous study (23).

The control group B displayed the classical picture of immunization which is seen after a single injection of LCM virus intraperitoneally into adult mice (24).

With regard to the somewhat surprising effect of the two control sera, CFA serum and NRS, both of which induced a prolonged viraemia and a delayed antibody response to the LCM virus, it is possible that the rabbit serum proteins have had a nonspecific suppressive effect on the immunocompetence of the mice (11, 12, 13), although other authors have not found depression of the humoral antibody production with normal rabbit serum (14). However, the effect is certainly not

as striking as that obtained with RAMLS treatment and the ultimate outcome of the virus inoculation seems to be immunization Experiments are in progress to demonstrate whether normal sera from other species have the same effect in the system under consideration In this connection it may be stated that preliminary results indicate that our anti lymphocytic serum is not strain specific this has also been shown by other authors (14)

Several previous studies have demonstrated that it is possible to produce anti lymphocytic sera which have a depleting effect on the leucocyte counts in the blood of experimental animals (1 5 16 25) Furthermore it has been shown that these sera can depress the immunological competence of these animals It is for example possible to prolong the survival of skin and kidney allografts considerably (1 14 25) even with a crude globulin fraction of antisera (14) In addition the primary response to sheep red cells can be depressed in mice and rats (11 14) but the effect on the secondary response is less striking

It would however seem to be impossible to induce a permanent tolerance to the hitherto used antigens even during prolonged treatment with anti lymphocytic serum Sooner or later after discontinuing the treatment the antigens (grafts) are rejected (1 13 14 15 25) However by combining anti lymphocyte treatment with drainage of the thoracic duct (25) or thymectomy (15) it may in a few cases be possible to induce permanent tolerance

During prolonged treatment with anti lymphocytic serum (> 4–5 weeks) complications to the treatment itself will often arise as evidenced by the appearance of infections (1) renal damage (10) or a wasting syndrome including liver necroses (14) In contrast in other experiments (25) the treatment is apparently found to be well tolerated Perhaps the discrepancies are caused by varying techniques used in production of the serum (11) and the use of different species of experimental animals

It is therefore of interest that the present investigations have demonstrated that 1) permanent tolerance to a self replicating antigen (LCM) can be induced with a high degree of regularity in adult mice treated with anti lymphocytic serum and 2) this treatment need [illegible] be of short duration (six days) We have in this way apparen[illegible]y avoided the unpleasant complications of prolonged anti lymphocyt[illegible] serum [illegible]reatment For the use of su[illegible]ra in human therapy it mu[illegible]t be [illegible]able to be able to reduce the [illegible]ation of the treatment as m[illegible]h as possible and still obtain the d[illegible] effect

The mode of actio[illegible] [illegible] lymphocytic sera is not [illegible] at the present time but it is impro[illegible] that it is du[illegible] to the redu[illegible]on in lymphocytes (1 10) It has be[illegible]n [illegible]sted (14) th[illegible] there may be two types of immunocompetent c[illegible] which have different sensitivity to anti lymphocytic serum One could imagine that the RAMLS sensitive cells were responsi[illegible] for transplantation immunity and virus elimina

24 Volkert M Larsen J Hannover & Pfau C J Studies on immunological tolerance to LCM virus 4 The question of immunity in adoptively immunized virus carriers Acta path et microbiol scandinav 61 268-282 1964
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Acta path et microbiol scandinav 71 481-501 1967

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THE NORMAL INTRAPULMONARY ARTERIAL PATTERN IN INFANCY AND EARLY CHILDHOOD

A Micro angiographic and Histological Study

By

BENGT ROBERTSON

Received 5 vi 67

This is the second part of a study on the pulmonary vasculature in the human foetus and infant As in the previous paper which was concerned with the pulmonary arterial pattern of the late foetal and neonatal lung (15) particular attention will be paid to various forms of aberrations from the basic arterial pattern *i e* to the occurrence of arterial bronchopulmonary anastomoses pulmobronchial arteries and focal bronchial artery supply of the pulmonary parenchyma This series will be extended to include comparative studies of congenital heart disease

PREVIOUS INVESTIGATIONS

The first report on the incidence of arterial bronchopulmonary anastomoses in early infancy was given in 1926 by *Konaschko* (6) who made corrosion casts of the bronchial and pulmonary arteries of human subjects from birth up to the age of one year Without stating their frequency *Konaschko* reported the normal occurrence of intrapulmonary and subpleural arterial bronchopulmonary anastomoses He also briefly mentions that the bronchial arteries normally supply small areas of the pulmonary parenchyma proper confirming an earlier observation by *Kuttner* (7)

Arterial bronchopulmonary anastomoses were also found in the normal infant lung by *Verloop* (19) who used serial sectioning and by *Marchand et al* (11) who used the corrosion cast technique and angiography these observations were however based on very few cases With injection and corrosion techniques *Tobin* (16) studied the arterial pattern of the foetal and postnatal human lung and found *arterial bronchopulmonary anastomoses though not consistently in*

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lung i e the branches of the pulmonary arteries generally follow those of the bronchi with the exception of the abrupt muscular branches from elastic pulmonary arteries (15) These branches are frequent in the medullary (circumhilar) part of the lung and are not accompanied by a bronchus at their points of origin

Some pulmonary arterioles leave their respective lobulus to supply either septal tissue or the visceral pleura These vessels run at the costal as well as at the mediastinal aspect of the lung and at the interlobar fissures Their diameter ranges from 25 to 250 μ A few of these vessels form arterial bronchopulmonary anastomoses (see below)

In one specimen from an infant aged two years and eight months a muscular pulmonary artery (diameter 500 μ) traversed the interlobular septum to ramify into an adjacent lobulus Pulmonary arterioles crossing interlobular septa were not encountered

Pulmobronchial arteries Pulmobronchial arteries [*rami pulmobronchiales* (2 4 5) *rami bronchiales arteriae pulmonalis* (25)] were demonstrated in small numbers in 8 subjects (53 per cent) Most of these arteries originated in elastic pulmonary arteries (Fig 1) but occasion

TABLE 2

Diameter Site and Wall Structure of 18 Pulmobronchial Arteries Demonstrated in Pulmonary Artery (PA) or Bronchial Artery (BA) Injected Lung Specimens from 8 Infants

Case No	Age		Injection of	Diameter (μ) of pulmobronchial artery	Sperr" artery structure	Site (lung lobe)
	Yr	Mth				
A 131		1	BA	125	—	LU
				75	—	LL
A 35		2½	PA	50	+	RL
A 132		5	BA	150	+	LL
A 99		7	BA	250	—	RL
A 33		10	PA	0	+	LL
				00	—	LL
A 14	1	1	PA	1 5	-	RM
				75	—	RM
				12	—	RM
				0	-	RM
				0		RM
A 11	1	4	PA	1	-	RL
				7	—	RL
				3 0		RL
				1	—	RL
					—	RU
A 133	1	8	BA	150	-	RL

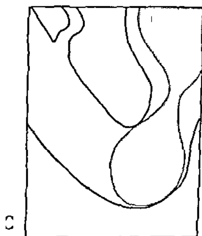


Fig 1

Ca 350 μ wide pulmobronchial artery (A white *arrow*) turning distally shortly after its point of origin (indicated by corresponding black *arrows* in A and B) Pulmonary artery type of mural elastic pattern before the artery joins the bronchial wall (B) Discontinuity of contrast medium in the bend but the vessel could be followed without interruption in the serial sections Age one year and one month

A Micro angiogram × 12

B Selected serial section from framed area in A Elastin van Gieson × 60

C Diagram of framed area in A showing the approximate level of B

series There remained then 25 lungs from 15 subjects in whom the injection was considered successful

Definition By Sperr artery is meant a muscular artery with mural thickening and an intimal layer of smooth muscle cells arranged longitudinally or irregularly (3-5 9 12 13 19 23)

RESULTS

Pulmonary Arterial System

Basic pattern The basic pattern of the pulmonary arterial system in early infancy does not differ from that of the late foetal and neonatal

lung i e the branches of the pulmonary arteries generally follow those of the bronchi with the exception of the "abrupt muscular branches from elastic pulmonary arteries (15) These branches are frequent in the medullary (circumhilar) part of the lung and are not accompanied by a bronchus at their points of origin

Some pulmonary arterioles leave their respective lobulus to supply either septal tissue or the visceral pleura These vessels run at the costal as well as at the mediastinal aspect of the lung and at the interlobar fissures Their diameter ranges from 25 to 250 μ A few of these vessels form arterial bronchopulmonary anastomoses (see below)

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Case No	Age Yr	Age Mth	Injection of	Diameter (μ) of pulmobronchial artery	"Sperr" artery structure	Site (lung lobe)
A 131		1	BA	125 75	— —	LU LL
A 35		2½	PA	50	+	RU
A 132		5	BA	150	+	LU
A 99		7	BA	250	—	RL
A 33		10	PA	250 200	+ —	LL LL
A 14	1	1	PA	12[illegible] 5 135 50 3 0	— — — — +	RM RM RM RM RM
A 11	1	4	PA	175 7 3 0 1 5	 - - — —	RL RL RL RL RL
A 133	1	8	BA	150	+	

ally they arose from intralobular pulmonary arterioles leaving their lobulus to join an adjacent prelobular bronchus The pulmobronchial arteries send concurrent as well as recurrent branches along the bronchi They can be distinguished from the true arterial bronchopulmonary anastomoses since their recurrent branches gradually decrease in size towards the hilus whereas the converse is true of the bronchial artery proximal to an anastomosis

The diameter of the pulmobronchial arteries at their point of origin ranges from 50 to 350 μ The mural structure of these arteries after they join the bronchial wall cannot be distinguished from that of the ordinary bronchial arteries Like the latter they often follow the peribronchial nerves Occasionally they have a thin subintimal layer of smooth muscle cells this feature was not observed before the age of 11 weeks The majority of the pulmobronchial arteries however do not have the morphological characteristics of Sperr arteries

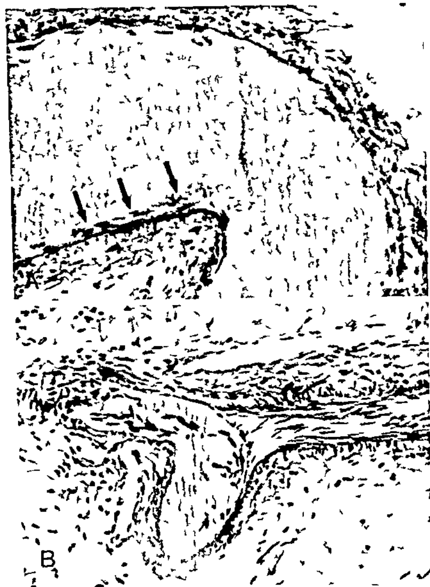
The inner diameter of the pulmobronchial arteries their site and wall structure are given in Table 2

Bronchial Arterial System

Basic pattern The bronchial arteries follow the course of the bronchi In the bronchial walls many branches of these arteries have precapillary intercommunications (*bronchial arterio arterial anastomoses*) Apart from the bronchial structures the bronchial arteries supply the lymph nodes of the lung They also send branches to the visceral pleura at the mediastinal aspect and the interlobar fissures of the lung Some of these pleural branches form arterial bronchopulmonary anastomoses This feature will be further discussed below The *vasae vasorum* of the major pulmonary vessels are derived from the bronchial arteries

Measured in micro-angiograms from bronchial artery injected specimens the inner diameter of the main bronchial arteries in the hilus of the lung ranges from 350 to 600 μ and does not increase significantly with age From the age of two months and a half longitudinal intimal muscle cells are present in the bronchial arterial system (Fig 2A) but the bronchial arteries are not uniformly involved Particularly affected are branches forming the *vasae vasorum* of the main pulmonary vessels (Fig 2B) and those participating in arterial bronchopulmonary anastomoses (see below) Even among the ordinary bronchial arteries there is a variation within the same specimen but as a rule the Sperr artery structure of intrapulmonary and pleural bronchial arteries becomes more prominent with age From the age of one year and four months some bronchial artery derived *vasae vasorum* of the pulmonary artery are completely obliterated by longitudinal smooth muscle cells (Fig 2B)

Bronchopulmonary arteries In 4 aorta injected and in 4 pulmonary



F 7
Sp rr rter es

A Layer of longitu linal smo th mu le ells (*arro vs*) inside the internal elastic membrane of a tortuous bro h l rt ry in an infant age two month and a half Ela t n–van Gieson × 210

B Longitudinal mooth muscl lls con letely ol literat ng a branch (*r ght*) of a bronchial artery derived *vas vasorum* in the adventitia of a major p lm nary artery Age one year and eigl t months Seri l section Ela tin van Gies n × 210



Fig 3

Small bronchopulmonary artery (A *arrow*) with circumscribed prominent narrowing of the lumen and thickening of the wall shortly after its point of origin The thick wall contains circular and longitudinal smooth muscle cells the latter cross cut in B The narrowed portion of the vessel is hidden in the micro angiogram (overlapping) Age one year and four months

A Micro angiogram × 25

B *Selected serial section showing the narrowed portion of the bronchopulmonary artery* Elastin van Gieson × 175

artery injected specimens *i e* 8 subjects (53 per cent) aged between 4 weeks and four years and seven months bronchial artery branches of different sizes (inner diameter $< 200\,\mu$) leave the bronchial walls to ramify as alveolar capillaries (Fig 3) In places contrast medium appeared to have passed from bronchopulmonary arteries to adjacent branches of the pulmonary artery through a common capillary network

At the age of 4 weeks bronchopulmonary arteries are fairly common *particularly in the medullary (circumhilar) part of the lung* From the age of 6 months these arteries are only rarely encountered in the micro angiograms Particularly near their points of origin in their parent bronchial arteries some of the small bronchopulmonary arteries are thick walled and narrow occasionally even obliterated by intimal layers of smooth muscle cells arranged longitudinally or irregularly (Fig 3) This Sperr artery structure of bronchopulmonary arteries was not observed until the age of 7 months The possibility of this appearance of obliteration being the result of an artefact due to tangential sectioning was excluded by serial sectioning furthermore obliterated arteries were also encountered in cross section Apart from these obliterated *arteries* the *mural* structure of the bronchopulmonary arteries after they enter the pulmonary parenchyma cannot be

distinguished from that of peripheral branches of the pulmonary artery Some of the bronchopulmonary arteries however are surrounded by a narrow sleeve of lymphoid tissue at their point of entrance into the pulmonary parenchyma A few bronchopulmonary arteries join small intralobular bronchi or bronchioles of corresponding size apparently substituting the pulmonary artery When present this direct bronchial artery supply of the pulmonary parenchyma proper consistently *only concerns a minute part of the lung*

Arterial Bronchopulmonary Anastomoses

True arterial bronchopulmonary anastomoses were demonstrated by serial sectioning in 12 subjects (80 per cent) Their number was about the same in all lung lobes but the number of anastomoses varied considerably from subject to subject They were particularly numerous in the two oldest subjects but there was no evidence of a gradual increase with age (Table 3)

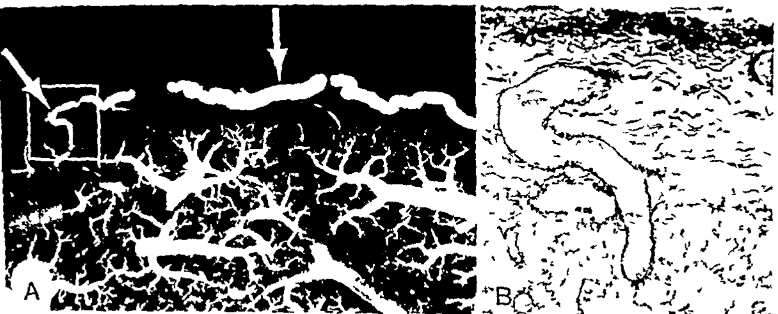
TABLE 3

Number Type and Size of Arterial Bronchopulmonary Anastomoses in Pulmonary Artery (PA) or Bronchial Artery (BA) Injected Lung Specimens from 15 Infants

Case No	Age		Injection of	Type of anastomosis			Total	Diameter range (μ)
	Yr	Mth		SS	ES	EE		
A 36		½	PA				0	
A 131		1	BA				0	
A 3		2½	PA	3	1	1	5	50-50
A 132		5	BA	8			8	25-200
A 99		7	BA		1	8	9	125-250
A 128		7	BA	9	2		11	0-275
A 33		10	PA	1		1	2	150-225
A 85	1		PA	3	2		5	75-275
A 14	1	1	PA				0	
A 11	1	4	PA	4	1	4	9	0-300
A 133	1	8	BA	5	2	1	8	50-225
A 101	1	8	BA	1			1	0
A 28	2	8	PA	1		2	3	0-150
A 43	3	8	PA	11	2	11	24	25-175
A 130	4	7	BA	9	2	11	22	0-200
Total				55	13	39	107	

0 = complete obliteration
SS = side to side ES = end to side EE = end to end

Side to side anastomoses Side to side (II) anastomosis is the most common type see Table 3 In this type of anastomosis a transverse (or oblique) vessel of varying length connects the more or less parallel branches of the bronchial and pulmonary arteries (Fig 4) Occasionally two or three bronchial artery branches will join to form the afferent systemic part of the II anastomosis (Fig 8) The side to side anastomoses are generally found in the walls of intrapulmonary prelobular



Fig

Pleural arterial bronchopulmonary anastomosis of end to end type in infant age two years and eight months The pleural bronchial artery (A *arrows*) *turns* and enters the pulmonary parenchyma anastomosing *with a peripheral pulmonary* arteriole (*framed area in A*)

A Microangiogram × 13

B Selected serial section *from framed* area in A Elastin-van Gieson × 83

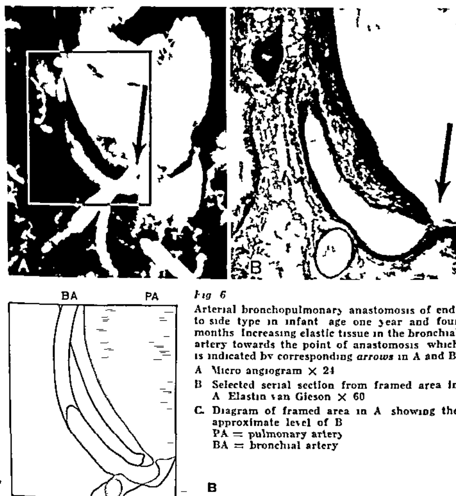


Fig 6

Arterial bronchopulmonary anastomosis of end to side type in infant age one year and four months Increasing elastic tissue in the bronchial artery towards the point of anastomosis which is indicated by corresponding *arrows* in A and B

A Micro angiogram × 24

B Selected serial section from framed area in A Elastin van Gieson × 60

C Diagram of framed area in A showing the approximate level of B
PA = pulmonary artery
BA = bronchial artery

End to side anastomoses The least common type is the end to side anastomosis in which an intrapulmonary bronchial artery empties into an elastic or muscular prelobular branch of the pulmonary artery (Fig 6 Table 3) The usual mural structure of the contributing bronchial artery is preserved until shortly before the point of anastomosis when the mural elastic pattern changes to pulmonary artery type The inner diameter of these anastomoses ranges from 25 to 250 μ (Table 5)

Obliteration of anastomoses From the age of two months and a half many of the bronchial arteries involved in arterial bronchopulmonary anastomoses have intimal layers of smooth muscle cells narrowing the lumen Generally this Sperr artery structure is more prominent in the bronchial arteries forming anastomoses than in the "ordinary



Fig 9

Organizing mural thrombus (*arrows*) in peripheral pulmonary arteriole running towards a pleural bronchopulmonary anastomosis of end to end type Age 1 month Elastin-van Gieson × 200

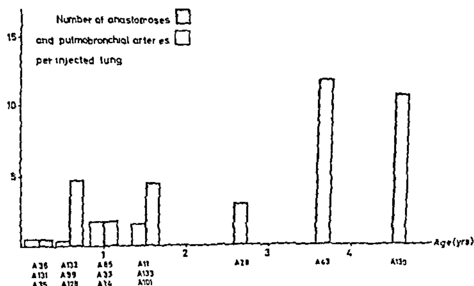


Fig 10

Diagram showing the relative incidence of arterial bronchopulmonary anastomoses and pulmobronchial arteries in postnatal lungs

least—result from the establishment of precapillary communications between pulmobronchial arteries and neighboring ordinary bronchial arteries

Another difference in the pulmonary arterial pattern between the late foetal neonatal period and early infancy is the postnatal development of pleural bronchopulmonary anastomoses and of pulmobronchial arteries derived from lobular penetrating pulmonary arterioles These structures were not demonstrated in the late foetal and neonatal lung (15) Concerning the pleural anastomoses they can be interpreted as a collision between the bronchial and pulmonary systems which normally share in the arterial supply of the visceral pleura The specimens however did not contain any actual evidence that this was the pattern of morphogenesis

As pointed out earlier (15) the micro angiographic technique offers a fairly complete screening of the arterial systems of the lung Anastomoses as well as the points of origin of pulmobronchial arteries are sometimes hidden however by close overlapping or missed at or near to the borderline between two slices of lung tissue This source of error inherent in the technique used may serve to explain why it proved impossible to demonstrate arterial anastomoses in three subjects in all of whom passage of contrast medium from one arterial system to the other was considerable suggesting precapillary communications Thus the observed incidences of pulmobronchial arteries (53 per cent) and arterial bronchopulmonary anastomoses (80 per cent) are probably lower than the true incidence of these structures

According to definition any recurrent branch of a pulmobronchial artery should decrease in size towards the hilus (15) This permits recognition of arterial bronchopulmonary anastomoses of the II type since the afferent bronchial artery gradually increases in size towards the hilus This distinction may be very difficult even impossible if the recurrent branch of a presumed pulmobronchial artery can be followed in the micro angiograms or in the serial sections for only a short distance and its diameter remains constant in its visible portion For the same reason a presumably pulmobronchial artery with nothing but recurrent branches can be hard to distinguish from a true arterial bronchopulmonary anastomosis of end to side type Finally pulmobronchial arteries originating in intralobular penetrating pulmonary arterioles resemble the peribronchial arterial bronchopulmonary anastomoses of end to end type and can be distinguished from these structures only if their branches run distally in the bronchial wall or if the recurrent branches th[at] may occur clearly diminish in size towards the hilus The wall structure *per se* is no clue to the origin of an arterial vessel in the bronchial wall the mural pattern of the pulmobronchial arteries after they join the bronchus cannot be distinguished from that of ordinary bronchial arteries Consequently there is a degree of uncertainty in the classification of pulmobronchial ar



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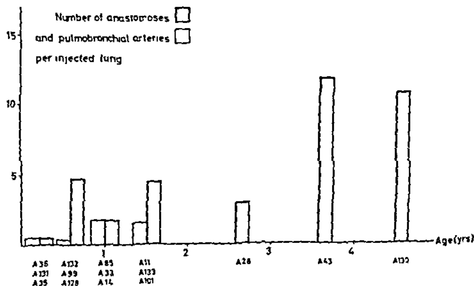


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enchyma proper (bronchopulmonary arteries) was present in one subject aged 4 weeks and was of about the same extension as that in the normal full term lung. In older infants the bronchopulmonary arteries were rarely demonstrated probably because many of these arteries become narrowed or completely obliterated by thick intimal bundles of smooth muscle cells (Sperr artery structure)

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TABLE 1

Distribution of Liver Biopsies According to histological Diagnosis and Identification of Periportal Limiting Plate with Indication of Patient's Sex and Age

Biopsy number	Histological diagnosis	Sex	Age	+ or — limiting plate
1463	posthepatitic cirrhosis	♀	73	—
1521	*biliary cirrhosis*	♂	73	—
1526	*nutritive cirrhosis*	♂	70	—
1548	hepatic steatosis	♂	64	—
1582	normal	♀	50	+
1583	normal	♂	19	+
1620	portal fibrosis	♂	51	—
1622	normal	♂	66	+
1634	acute hepatitis	♀	71	+
1640	normal	♀	71	+
1656	normal	♀	71	+
1663	portal fibrosis	♂	46	+
1668	hepatic steatosis	♂	42	+
1683	hepatic steatosis	♀	75	—
1684	hepatic steatosis	♂	78	+
1698	normal	♀	46	+
1699	*nutritive cirrhosis*	♂	65	—
1701	*toxic hepatitis*	♂	39	+
1702	*acute hepatitis*	♀	50	+
1705	hepatic steatosis	♂	68	+
1709	hepatic steatosis	♂	42	+
1718	carcinoma	♂	72	—
1766	normal	♀	72	+
1767	cirrhosis	♀	44	+
1769	normal	♂	21	+
1770	acute hepatitis	♀	44	+
1776	metastatic carcinoma	♂	70	+
1777	cirrhosis	♂	55	—
1799	*acute hepatitis*	♂	73	+
1800	*normal*	♀	65	+
1808	*normal*	♂	58	+
1809	*metastatic carcinoma*	♀	61	—
1811	acute hepatitis	♂	50	+
1812	normal	♂	52	+
1816	carcinoma	♂	63	—
1821	metastatic carcinoma	♂	48	—
1824	hepatic steatosis	♂	35	+
1837	acute hepatitis	♂	30	+
1846	hepatic steatosis	♂	58	+
1861	posthepatic cirrhosis	♀	81	—
1871	*acute hepatitis*	♂	64	+
1890	posthepatic cirrhosis	♀	61	—
1894	*normal*	♀	72	+
1901	acute hepatitis	♂	63	+
1904	acute hepatitis	♂	70	+
1907	hepatic steatosis	♀	82	+
1915	normal	♀	82	+
1921	toxic hepatitis	♀	75	+
1929	portal fibrosis	♂	44	+
1945	*hepatic steatosis*	♀	72	+
1950	amyloidosis	♀	50	+
1959	acute hepatitis	♀	63	+
1971	posthepatic cirrhosis	♀	78	+

TABLE 2

Distribution of PAS Positive Material in Cells of Periportal Limiting Plate

Histological diagnosis		PAS positive material in cells of limiting plate
Normal	1582	+
	1583	inferior quality of slide
	1692	+
	1640	+
	1656	+
	1698	+
	1766	inferior quality of slide
	1769	+
	1800	+
	1803	+
	1812	+
	1894	+
	1915	+
Hepatic steatosis	1668	+
	1684	+
	1705	+
	1709	+
	18 4	+
	1846	+
	1905	+
	1945	+
Cirrhosis	1767	+
	1971	+
Hepatitis	1634	+
	1701	+
	1702	+
	1770	+
	1799	+
	1811	+
	1837	+
	1871	+
	1901	+
	1904	+
	1991	+
	19 9	+
Liver with carcinoma	1776	not enough tissue to identify limiting plate
Portal fibrosis	1663	+
	1999	+
Amyloidosis	19 0	+

Schaffner have as a referenc *H ser* (193) who however describes that administration of insul   to normal animals gives rise to a deple tion of the liver glycogen T   glycogen content of the liver cells de creases from the central zor   to the periportal zone of the lobule and glycogen is often complete   bsent from th   cells of the periportal li miting plate Fasting also r sults in a depletion of the liver glycogen especially from the periportal zone of the lobule as shown by *Ekman &*

TABLE 1

Distribution of Liver Biopsies According to histological Diagnosis and Identification of Periportal Limiting Plate with Indication of Patient's Sex and Age

Biopsy number	Histological diagnosis	Sex	Age	+ or — limiting plate
14[illegible]3	posthepatitic cirrhosis	♀	73	—
1521	biliary cirrhosis	♂	73	—
1526	nutritive cirrhosis	♂	70	—
154[illegible]	hepatic steatosis	♂	64	—
1582	normal	♀	50	+
1583	normal	♂	19	+
1620	portal fibrosis	♂	51	—
1[illegible]22	normal	♂	[illegible]6	+
1634	acute hepatitis	♀	71	+
1640	normal	♀	71	+
1656	normal	♀	71	+
1663	portal fibrosis	♂	46	+
1[illegible]6[illegible]	hepatic steatosis	♂	72	+
1[illegible]83	hepatic steatosis	♀	75	—
1684	hepatic steatosis	♂	78	+
169[illegible]	normal	♀	4[illegible]	+
1699	nutritive cirrhosis	♂	[illegible]5	—
1701	*toxic hepatitis*	♂	3[illegible]	+
1702	acute hepatitis	♀	50	+
1705	hepatic steatosis	♂	68	+
1709	hepatic steatosis	♂	42	+
1718	carcinoma	♂	72	—
17[illegible]6	normal	♀	72	+
1767	cirrhosis	♀	44	+
1769	normal	○	21	+
1770	acute hepatitis	♀	44	+
1776	metastatic carcinoma	♂	70	+
1777	cirrhosis	♂	[illegible]5	—
179[illegible]	acute hepatitis	♂	73	+
18[illegible]0	normal	♀	6[illegible]	+
1808	normal	♂	58	+
1809	metastatic carcinoma	♀	61	—
1811	acute hepatitis	♂	50	+
1812	normal	♂	5[illegible]	+
181[illegible]	*carcinoma*	♂	[illegible]3	—
1821	metastatic carcinoma	♂	5[illegible]	—
1824	hepatic steatosis	♂	3[illegible]	+
1837	acute hepatitis	♂	30	+
1846	hepatic steatosis	♂	58	+
18[illegible]1	posthepatic cirrhosis	♀	81	—
1871	*acute hepatitis*	♂	[illegible]4	+
188[illegible]	posthepatic cirrhosis	♀	61	—
1894	normal	♀	[illegible]	+
1901	acute hepatitis	♂	[illegible]3	+
1904	acute hepatitis	♂	70	+
190[illegible]	hepatic steatosis	♀	8[illegible]	+
191[illegible]	normal	♀	82	+
19[illegible]1	toxic hepatitis	♀	25	+
19[illegible][illegible]	portal fibrosis	♂	44	+
1945	hepatic steatosis	♀	72	+
19[illegible]0	amyloidosis	♀	50	+
19[illegible]9	acute hepatitis	♀	63	+
1971	posthepatic cirrhosis	♀	78	+

TABLE 2

Distribution of PAS Positive Material in Cells of Periportal Limiting Pl[illegible]e

Histological diagnosis		PAS positive material in cells of l[illegible]t[illegible] plate
Normal	158[illegible]	+
	1583	inferior quality of slide
	16[illegible]	+
	1640	+
	1656	+
	1698	+
	1766	inferior quality of slide
	1769	+
	1800	+
	1808	+
	181[illegible]	+
	1894	+
	1915	+
Hepatic steatosis	1668	+
	1684	+
	1705	+
	1709	+
	18[illegible]4	+
	1846	+
	1905	+
	1945	+
Cirrhosis	1767	+
	1971	+
Hepatitis	1634	+
	1701	+
	170[illegible]	+
	1770	+
	1799	+
	1811	+
	1837	+
	1871	+
	1901	+
	1904	+
	1921	+
	1959	+
Liver with carcinoma	1776	not enough tissue to identify limiting plate
Portal fibrosis	1663	+
	1929	+
Amyloidosis	1950	+

Schaffner have as a reference *Hanse* (1930) who however describes that administration of insulin to [illegible]rmal anim[illegible]ls giv[illegible]s rise to a deplection of the liver glycogen The glycog[illegible]n content of the liver cells decreases from the central zone to the periportal zone of the lobule and glycogen is often completely a[illegible] [illegible] fr[illegible]m the [illegible]ls of the periportal limiting plate Fasting also res[illegible]ts [illegible] a depletion of the liver glyco especially from the periportal zone [illegible] he lobule as shown b



Fig 1

Section from biopsy No 1894 (normal) stained for PAS positive material Nuclei counterstained with Mayer's haemalum × 140



Fig 2

Section adjacent to section shown in Fig 1 stained for PAS positive material after prior treatment with diastase Nuclei counterstained with Mayer's haemalum × 140

Holmgren (1949) This is in accordance with *Chiquoine's* (1953) demonstration that the concentration of glucose 6 phosphatase that mediates the mobilization of glycogen as glucose is greatest in the periportal zone

It thus seems that the cells of the periportal limiting plate have a certain content of glycogen under normal conditions but that they rapidly lose this during prolonged fasting or following administration of insulin Our results are in accord with this statement The patients have as a rule fasted for about twelve hours prior to biopsy This period of time would not be sufficiently long to deprive the cells of the periportal limiting plate of glycogen

The biopsies have primarily been performed with the object of studying the distribution of enzyme activity and the method of fixation has therefore not been especially designed for the optimal preservation of glycogen *Pearse* (1960) states that with the possible exception of Bouin's fixative the formalin containing fixatives in routine use are more or less objectionable from the histochemical point of view Also *Spicer* (1958) has shown that aqueous solutions of formalin give inferior histological preservation of glycogen However *Lillie* (1965) states that neutral aqueous formalin solutions often preserve glycogen quite well especially in fresh animal livers but the action of certain acid alcholic fluids is more reliable In our material the main objective was to determine whether there was glycogen in the cells of the periportal limiting plate This has been shown to be the case and the degree of glycogen preservation seems to be adequate from a histological point of view An analysis of the distribution in the individual liver cells would however not be reasonable taking the above mentioned findings into consideration

SUMMARY

A light microscopic study of glycogen in the cells of the periportal limiting plate in human biopsy material normal and pathological is presented

In the literature it is often stated that these cells are devoid of glycogen We have not been able to confirm this and have found glycogen in the cells of the periportal limiting plate in both normal and pathological conditions

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The University Institute for Experimental Medicine (Head: Jens Bing, M.D.), Copenhagen, Denmark

RENIN LOCATION IN THE DIFFERENT PARTS OF THE JUXTAGLOMERULAR APPARATUS IN THE CAT KIDNEY

I. The Afferent Arteriole and the Macula Densa

By

POUL FAARUP[1]

Received 13.vi.67

In recent years the location of renin in the kidney has been investigated by different micro fractionation techniques as well as with the use of qualitative immunological methods (*Hartroft & Edelmann* 1961). The renin content in different fractions of the nephron has been studied in cryostat sections from micro dissected tissue (*Bing & Wiberg* 1958) from kidneys which in some cases had been previously perfused with an ammonium sulphate solution (*Bing & Kazimierczak* 1959, 1960, 1962). *Cook & Pickering* (1959) combined a rough fragmentation of kidney tissue with the introduction of microscopic iron particles into glomeruli which could then be separated out in a magnetic field. Similar preparations were later microdissected (*Cook* 1960). With the aid of such techniques renin has been found to be confined to the juxtaglomerular apparatus of the nephron.

However, the exact localization of renin in the juxtaglomerular apparatus is still unsettled. Thus some of the investigations performed with the use of fluorescent antirenin would indicate that renin is only found in the granulated cells of the vessel (*Hartroft & Edelmann* 1961, *Hartroft* 1963, *Hartroft et al.* 1964). Contrary to this, other studies in which a similar technique was applied (*Warren et al.* 1966) as well as the above mentioned microdissection experiments on ammonium sulphate perfused kidneys would speak in favour of the interpretation that renin is located in both the afferent arteriole and in the macula densa.

In the present as well as in the following paper the renin content of the different parts of the juxtaglomerular apparatus was determined after the isolation of these cell groups by means of a micromanipulator.

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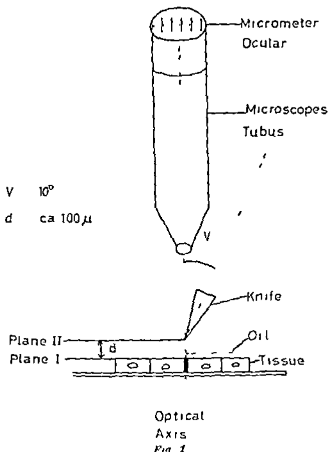


Fig 1

Diagram showing the course of action in the microdissection of the freeze dried tissue The section was placed in such a way that the plane of section would be par llel to th edge of the knife and would cross the optical axis of the micros ope Secondly the knife was adjusted to lie ca 100 microns above the tissue (plane II) the edge of the knife being placed in the optical axis Finally the knife was lowered into and through the tissue The knife blade was tilted about 10 (V) from the vertical to allow the microscope to be focussed onto the edge of the knife

artery forceps in which a small piece of r r Bla l (t p e Gillette Blu) wa pla ed This was used as a knife as the temper f th s tvp f blade makes the metal br ttle while the edge of the knife will not det rm hen h blade is fractured The angle betw een the plane of the knife an l th rti al wa bout 10 (V in Fig 1) as the accurate positioning of the knif re q ires that its edge be clea ly seen in the microscope

The microscope used for the m sectio n wa a Zeiss St ndard GI l micro scope with a total magnification f × 200 (obje tive × 10 cular × 20) The normal stage of the microscope w eplace l by one in h ch circular movement of the bject could be made

F r micro lissection *the a ljustment of the mi rom nipulator* was perform d in the following step

1) In the plane of the micr s pe stage (plan I in Fig 1) the fre ze l tion was placed in a small amount f paraffin cil upon a sli le By u e f ter o ula the tis ue was positione l in the optical axis of the mi rosc p ro

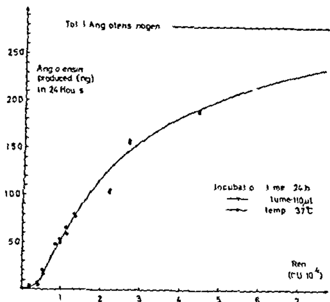


Fig 2

Incubation of substrate together with various concentrations of Goldblatt renin The microdissected fractions of the tissue were incubated in three different renin concentrations of which at least one gave less than 30 per cent transformation of the substrate *which in this case contained about 280 ng of potential angiotensin*

tated so that the required plane of section in the tissue was parallel to the edge f the knife of the micromanipulator

2) The knife was now placed beneath the objective of the microscope an l the e lk of the knife was adjusted in the optimal axis of the microscope and somewhat al the object plane by using the micrometer ocular The final a ljustment was done it the smallest possible distance from the plane of the object This was about 100 micron (plane II in Fig 1)

3) The knife was lowered vertically into the tissue thus producing the requir l section

4) That the separation desired was obtained was checked in another mierose je (type Reichert Zetopan) at a magnification of × 400 Subsequently fragments w isolated from the rest of the tissue by manual dissection using a piece of raz r l la ie *held in an artery forceps*

The accuracy of the microdissection with the micromanipulator was *in the rank* of 1 *micron* b hand it was 10–15 micron Within this *limit of accuracy using* t e micromanipulator it was possible *to carry* out the dissection of the tissue in t e *following* ways

I) At the boundary between the afferent arteriole and the macula densa (I ig ı)

II) Just basal to the nuclei of the cells in the macula densa a *small part of* t e cytoplasm from these cells being in the vascular fraction

III) In the middle of the cells *of the macula densa* The basal half of the cells would thus be included in the vascular *fraction* (I ig 4)

In experiments in which the last two methods were used the number of cells fr m the macula densa taken in each *sample* (× 1 25 an l × 2 respectively) v as higher than that needed in the first metho l

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The working principles *here described may* be used with any micromanipulator provided that a separate indirectly *operated* vertical *movement* of high precision is present An apparatus of *this type is manufactured* by Leitz Ltd. in Germany The micromanipulator used in the present work was constructed at our institute

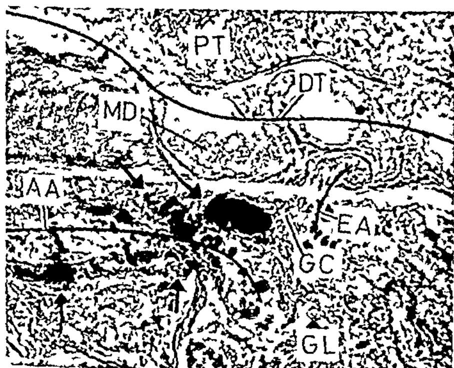


Fig 3

In the juxtaglomerular apparatus the macula densa (MD) has been eparated from the glomerulus (GL) the cell group of Goormaghtigh (GC) and the afferent arteriole (AA) in which several cells containing juxtaglomerular granules (arrows) are found in the distal part The efferent arteriole (EA) was removed from the macula densa before renin as as DT distal tubule PT proximal tubule (Freeze dried section vitally stained with Neutral Red × 350)

C. Perfusion with a Mixture of Ammonium Sulphate and Light Green

Three kidneys (the left kidney from nos 9 10 and 15) were perfused through the renal artery with a solution containing 9 parts of saturated ammonium sulphate and 1 part of a 1 per cent solution of Light Green as pre iousl used by *Bing & Ka i mi re al* (1960 19 2) In one kidney (f m at no 9) in which the perfusion was done by hand the perfusion pressur is not measured With the other kidneys (f om nos 10 and 15) a perfusion pre ure 10–20 mm mercury lower than the mean blood pressure was used (90 and 1?0 mm respe tively) For all kidneys the per fusion time was 8–10 min Ti sue fro th renal cortex was frozen in isopentane at ab ut —160 C and sections of 100 ron thi kness w re cut in the cryo tat and stored in a solution of saturated a m nium sulphate t 4 C until the microdi sec tion was made Just prior to the f tionation the se tions were counterstained with a solution of 1 pe cent eo in turated ammonium sulphate In th s way the tubules and the glomeruli v re tained red in contrast to the green colour of the perfused vessels The separati n of the affer nt arter ole from the distal tubule including the ma ula densa v done by us of a blunt technique rre pon ling to that emplo ed by *Bing & K r terc al.* (1960 196?) with the exception that in the region in which the tru t res a e t ghtly connected the afferent art ri le was separate l from the m a l lensa by means of a piece of az r bla le f xe l in an artery forceps The affer nt rteriole and the macula densa region of the di tal tubule from 3 5 sul apsul juxtaglomerular apparatu es were pooled for the e timation

The perfusate from tl 3 ammonium sulphate perfuse l kidne was between 10

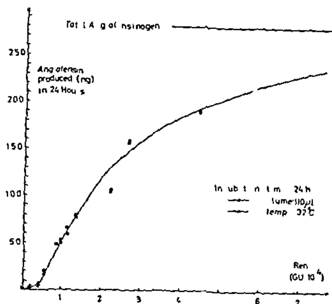


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(pH 7 5) and 1? µl of 2 N HCl and boiling for 10 minutes Following centrifugation at 4000 g 12 µl of 2 N NaOH were added to the supernatant The samples were sto ed at −20 C until they were assayed

The assay was carried out in rats of about 200 g anaesthetized with amytal (125 mg/kg) and pretreated with a ganglion blocking agent (pentolinium 8 mg/kg) Angioten in was estimated by comparison with angiotensin amide (Hypertensin Ciba) at concentrations of 100 ng/ml and 40 ng/ml

3) *Renin estimation in extracts from the microdissected tissue sections* The ex traction of the isolated fractions of the juxtaglomerular apparatus was performed at 4 C for 10 minutes at 600 rpm in a glass homogenizer containing 200–300 µl substrate and 20 µl toluol Next, the solution was centrifuged for 10 minutes at 15 500 g The incubation of the upernatant was done as described in *section 2 (standard curve)* and each extract to be assayed was incubated in three different dilution with a constant concentration of substrate thus enhancing the accuracy of the renin estimation Prior to the incubation 10 µl of Tris buffer (pH 7 5) was added so as to make the substrate concentration in the samples to be tested identical with that of the samples from the standard curve

As it was found that the small content of ammonium sulphate in the micro dissected fractions from the kidneys perfused with ammonium sulphate and Light Green did not interfere with the renin activity of the samples dialysis of the e frac tions could be omitted

Assay of angiotensinase like activity in the samples By the technique use l which comprised a 24 hours incubation with angiotensin amide (Hypertensin Ciba) no angiotensinase activity could be demonstrated in either the tissue fractions or in the Goldblatt renin in the amounts used

Assay of angiotensin like pressor activity in the microdissected tissue By incubat ing preparations of the juxtaglomerular apparatus from the freeze dried sections in the absence of sub trate no angiotensin like pressor activity was measurable in the sample which were quantitatively equivalent to those used for renin estimation as previously described The same was found by *Brown et al* (1965) who investigated whole glomeruli with attached juxtaglomerular apparatus

Isolated Fractions of the Cortex Corticis for Renin Estimation

For an estim tion of the renin content of the cortex corticis a 200 micron deep zone was isolated from freeze dried sections of kidneys which were vitally stained with Neutral Red (from cats nos 9 and 15) as well as from cryostat sections of kidn ys perfused with a mixture of ammonium sulphate and Light Green (from cat nos 9 10 and 15) Following dialysis for 24 hours and extraction of the tissue the samples were incubate l using the method described by *Poulsen* (1957) which is base l up n an estimation of the content of angiotensinogen before and after the incubation and is therefore not influenced by the angio tensinase activity present in the quantity of the tissue used (Table 3)

Estimation of the Renin Content in the Cortical Tissue of the Kidney by the Direct Metho l

The renin content in extracts of pieces of the kidney cortex was tested in ats anaesthetize l with amytal and pretreate l with 0 007 mg ergotamine In the three animal in which the one kidney was perfused with an ammonium sulphate solution homogenized tissue from this as well as from the opposite kidney was dialysed for 24 hours before the renin extraction The renin content was expressed n GU p r g total cortex

Control experiments have demonstrated that the renin content of the kidney ortex was unchanged by the freeze drying procedure

Histological Examination

1 *Morphological evaluation of th fra t ons of the juxtaglomerular apparatus from free I ied sections* In each of t test fractions the number of cells containing juxtaglomerular granules cells i he macula densa an l cells belonging to the cell group of Goormaghtigh was cour d By means of this analysis it was possible to convert the renin content in the te t samples to that which would have been found in an extract of a "standard juxtaglomerular apparatus (Table 1) The number of

cells in such a representative standard was established from a cell count made on 5 subcapsular juxtaglomerular apparatuses from two kidneys vitally stained with Neutral Red and investigated in 25 micron thick freeze dried sections

It is seen from Table 1 that the number of cells in the macula densa showed a two fold maximal variation and the number of granulated cells and of the cells from the cell group of Goormaghtigh varied up to 3 3 × The maximal deviation from the mean value was 1 5 × 2 1 × and 2 0 × respectively

In one of the juxtaglomerular apparatuses investigated by the serial sectioning technique the efferent arteriole was found to contain a single lightly granulated cell

II *The Juxtaglomerular Index* was investigated in Bowie stained sections from kidneys (*Hartroft* & *Hartroft* 1953 1955) which had not been perfused with ammonium sulphate

TABLE 1

Number of Cells Counted in the Different Fractions of Each of Five Subcapsular Juxtaglomerular Apparatuses

Cat no	12	12	13	13	13	Mean value per fraction
Granulated cells in afferent art	20	24	20	9	8	17
Cells in macula densa	41	53	44	30	25	39
Goormaghtigh cells	10	9	3	3	6	6

The mean values were used when the renin content in different fractions from the freeze dried tissue were compared Thus the estimation was rendered independent of the variations in the number of cells occurring in the test fractions

RESULTS

The Renin Content of the Afferent Arteriole and of the Macula Densa in Subcapsular Nephrons

1 *Freeze dried tissue* In Fig 2 it is seen that the renin content of the vascular fraction was always found to be greater than that of the fractions containing the macula densa as in the last case the renin content was measurable ($\geqq$ 0 4 × 10^{-4} GU) in 7 only out of 20 preparations In the Neutral Red stained preparations in which the macula densa was separated from the afferent arteriole at the boundary between the two structures a measurable renin content was found in out of 13 samples (Fig 3) In 5 preparations where the separation was done inside the macular cells (Fig 4) it was only possible to demonstrate the presence of renin in the 2 unstained preparations In two cases where the macula densa had been isolated from areas where a relationship was found only to the *efferent arteriole* the renin content was not measurable

The average renin content of the extracts of the *afferent arteriole* taking all the fractionations performed into account was 16 1 × 10^{-4} with great variability between the different animals the values varying between 47 4 × 10^{-4} GU (no 17) and 1 4 × 10^{-4} GU (no 8) based on *equal numbers of granulated cells* (Table 2) It appears from the table that the renin content per afferent arteriole from the same kidney

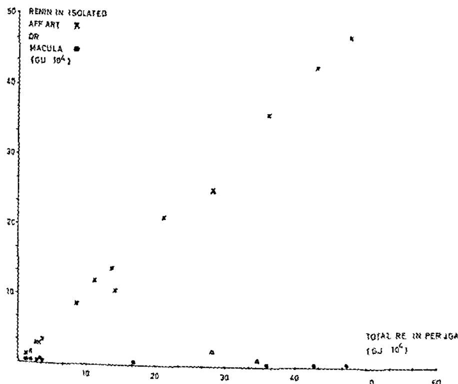


Fig 5

The renin content of the afferent arteriole and of the macula densa in freeze dried tissue In 13 cases out of 20 the renin content of the macula densa fraction was not measurable *Abscissa* Total renin content in both the afferent arteriole and the macula densa *Ordinate* Renin content of the single fractions × Afferent arteriole ● Macula densa ▲ Fractions of the macula densa the separation of which from the afferent arteriole was carried out through the cytoplasm of the macula cells basally to the nuclei ▼ Fraction of the macula densa the separation of which from the afferent arteriole was done in the middle of the macula cells (cf Fig 4) ■ Macula densa separated from the rest of the juxtaglomerular apparatus in places where it was adjacent to the *efferent* arteriole only and where no juxtaglomerular granules were found in the vessel Filled symbols Neutral Red stained tissue Open Symbols Unstained tissue

varied up to 2 6 × whereas the maximal variation between different kidneys was 28 ×

The upper and lower limits for the renin content of the *macula densa* were $3\ 6 \times 10^{-4}$ GU (no 9) and less than $0\ 4 \times 10^{-4}$ GU (nos 6 7 8 and 15) From all the tests made the average renin content of the macula densa was less than $0\ 7 \times 10^{-4}$ GU corresponding to less than 4 per cent of the total renin content of the juxtaglomerular apparatus In Fig 5 it is seen that the variation in the quantity of renin in the afferent arteriole was not accompanied by a corresponding variation in the renin content of the macula densa

2 *Tissue perfused with ammonium sulphate Light Green solution* Table 2 and Fig 6 show that the renin content of the *afferent arteriole*

opposite kidney in the same animal was almost identical (nos 9 10 and 15) In agreement with this the perfusates from the perfused kidneys contained only insignificant quantities of renin (1 GU in each)

No close relationship was found between the renin content of the kidney cortex and the juxtaglomerular index in those kidneys where both parameters were investigated (Table 4)

TABLE 4

The Renin Content in Whole Cortical Tissue per g in Goldblatt Dog Units and the Juxtaglomerular Index in the Microdissected Kidneys

Cat no	Renin in GU/g kidney cortex	Juxtaglomerular index	
6 (l)	20	18	b
7 (l)	–	19	b
8 (l)	3	25	b
9 (r)	19	20	b
(l)	21		c
10 (r)	10	19	a
(l)	9		c
12 (l)	10	47	a
15 (r)	11	31	b
(l)	14		c
16 (l)	14	19	b
17 (r)	10	17	b

a unstained tissue b Neutral Red stained tissue and c ammonium sulphate Light Green perfused tissue r right kidney l left kidney

SUMMARY AND CONCLUSIONS

The location of renin in the granulated cells of the afferent arteriole and of the macula densa of the juxtaglomerular apparatus was investigated both in freeze dried kidney sections and in cryostat sections from kidneys previously perfused with an ammonium sulphate solution

In the freeze-dried tissue the average quantity of renin present in the macula densa was less than 5 per cent of that found in the afferent arteriole whereas in the ammonium sulphate perfused preparations the renin content of the two fractions was found to be almost identical

The difference observed in the two preparations may well be explained by a dislocation of renin at the ammonium sulphate perfusion procedure as indicated both by a diminution in the renin content of the afferent arteriole and by the appearance of a slight renin activity in the cortex corticis of 2 of the 3 kidneys perfused with ammonium sulphate

The results obtained thus speak in favour of the conception that renin is exclusively or predominantly localized in the granulated epithelioid cells

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12 (l)	10	47	a
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MEASUREMENT OF CARBON UPTAKE BY THE RETICULO ENDOTHELIAL SYSTEM

By

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Received 7.vi.67

In 1951 *Halpern et al* (2) introduced the speed of removal of carbon particles from the blood stream as a measure of the phagocytic activity of the reticulo endothelial system Using this method blood is withdrawn at timed intervals (a & b) after the intravenous injection of colloidal carbon and the concentration of carbon determined spectrophotometrically The phagocytic index K is then calculated from the formula (1)

$$K = \frac{\log A - \log B}{b - a}$$

where A is the concentration of carbon in the blood at the time a shortly after injection and B the concentration at a later time b

The present study demonstrates a simpler method of measuring the clearance of carbon particles from the blood by direct microscopical observation of a dried blood drop The time of disappearance of carbon particles is thus determined directly Simultaneous results recorded by both methods are compared

MATERIAL AND METHODS

Mice Adult male mice of the closed colony kept at this Institute were used Their mean weight (± S D) was 30 3 ± 5 0 g *Carbon particles* *Pelikan* ink (Gunther Wagner Hanover) was used. This ink contains 100 mg carbon/ml the particle size varying from 200–500 Å A 20 per cent suspension of ink in physiological saline containing 1 per cent gelatine as stabilizing agent was used for injection

The carbon suspension was injected into the tail veins Thereafter blood samples were taken off at intervals timed to the nearest minute

Samples of blood were examined *spectrophotometrically* by *Halpern et al's* (3) method as follows

Samples of 0 05 ml of blood were taken from the retro orbital plexus into graduated heparinized tubes The sample was then mixed with 4 ml 0 1 per cent Na_2CO_3 to disrupt the red blood cells The resultant mixtures were examined spectrophotometrically (Unicam spectrophotometer) the extinction value at 650 mμ being recorded using a specimen with carbon free blood as blank

Samples of blood were examined microscopically as follows

The tail tip (ca 1 mm) was cut off and a small drop of blood allowed to collect

against time in mice given 8 mg carbon/100 g body weight At the same time the corresponding microscopical readings are recorded as positive or negative Extinction values of 0 060 mμ and over gave positive readings while those of 0 056 mμ and under gave negative Thus a clear borderline can be drawn between positive and negative readings

Parallel microscopical readings on blood from the tail and the retro orbital plexus were available in these mice The results were identical Thus back flow from the injection site has not occurred

TABLE 1

The Intervals between Taking Blood Sampl s for Microscopic and Spectrophotometric Evaluation Related to the Number of Mice Used and the Carbon Dosage

No of mice	Carbon dosage (mg/100 g)	Time between micro samples (mins)	Time between spectro samples (mins)
17	16	5	15
10	8	10	10§
6	4	2	10

repeated until negative
§ repeated at 10 minute intervals for 1 hour first and second readings used for K

TABLE 2

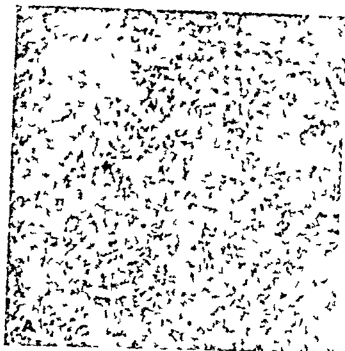
The Carbon Content of Mouse Blood on Serial Dilution Measured Microscop cally and Spectrophotometrically (see Text)

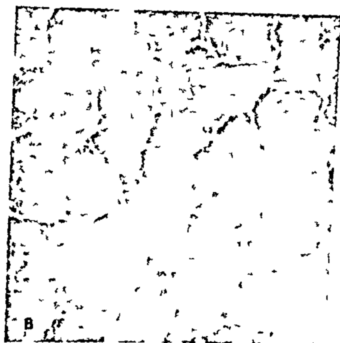
Dilution	Microscopical reading + present 0 absent	Spectrophotometric extinction value (mμ)
1/1	+	0 315
1/2	+	0 190
1/4	+	0 100
1/8	0	0 050
1/16	0	0 0?7
1/3?	0	0 013

Comparison of Results of in vivo Exp   m nts

Table 3 compares the mean K value (± S D) determined microscopically after 16 8 and 4 mg carl c n   00 g body weight The K value which is equivalent to the speed of clearance of carbon particles from the blood stream increased with increasing dosage This is in keeping with *Bio. t et al* s findings (1) Microscopic determination of clearance time showed the same pattern the speed of clearance increased with decreasing dosage

Table 3 thus shows a negative correlation between the mean K values and the mean microscopical clearance time Further analysis of these





Figs 24 A & B

Microscopic view of blood drop containing carbon particles (A) and drop free of carbon particles (B) (× 128) Note thickness of drop used as seen from mosaic pattern

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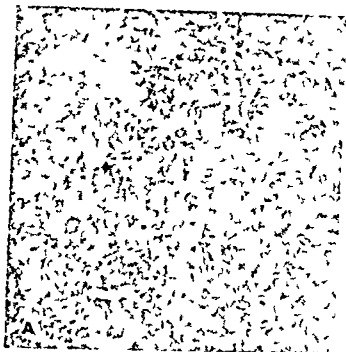
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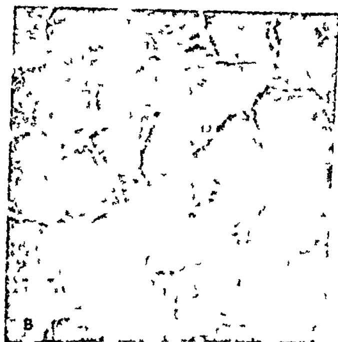
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Figs ?A & *B*

Microscopic view of blood drop containing carbon particles (A) and drop free carbon particles (B) (× 1?3) Note thickness of drop used as seen from mosaic pattern

mean The greatest deviation (51 per cent) was recorded following spectrophotometry at a dose of 16 mg The deviation dropped to about half after 8 and 4 mg Following microscopic reading a deviation of 24 per cent was recorded after 16 mg while the values were higher after 8 and 4 mg

DISCUSSION

Halpern et al (2) have shown that the phagocytic activity of the reticulo endothelial system in mice can be measured by determining the speed of disappearance of carbon particles from the blood stream following intravenous injection the concentration of carbon being measured spectrophotometrically The present experiment shows that it can also be measured directly microscopically

Colloidal carbon is easily seen microscopically at a magnification of 198 times in dried mounted blood drops Mounting is essential to exclude air which masks the carbon particles *In vitro* dilution tests showed that the limit of microscopical resolution of carbon particles at this magnification lay between concentrations giving extinction values of 0 100 mμ and 0 050 mμ This finding is supported by the *in vivo* results which further define the borderline to concentrations giving an extinction of under 0 060 mμ but over 0 056 mμ As complete correlation was found in the results of microscopical readings taken on blood from the tail and from the retro orbital plexus the difference in sampling site does not effect the results

The experiment shows that there is a highly significant correlation between the results obtained from the two methods This is to be expected as they both represent different methods of measuring the same phenomenon disappearance of carbon from the blood stream Lack of correlation will thus be due to the experimental errors involved in the two methods

Experimental error can be judged in part from the deviation of the results from their mean While the basic variation due to biological causes can not be cut down variation due to experimental error can be

The spectrophotometric method presents many opportunities for experimental error to creep in and the variation recorded is correspondingly high especially when high carbon dosage is used The latter may be the result of recording values over the first half of the clearance time (as judged microscopically) although Biozzi et al's results (2) which give a linear relationship between time and log concentration of carbon imply that the time of the second reading should be arbitrary within the limits of the dosage used In the present case when the second values were taken at times nearer the microscopic clearance time progressively less variation was obtained

The lowest variation obtained with the microscopical readings was after a dose of 16 mg/100 g body weight These readings were taken-

at 5 minute intervals The variations at lower doses were greater The reason probably is that 10 minutes was too great an interval at 8 mg dosage while the concentration dropped so fast after 4 mg that even 2 minutes was too great at that dosage It thus seems that while it is possible to obtain less variation in results with the microscopical method care should be taken to adjust the time between readings to the dosage used The major part of the experimental error involved in the microscopical method appears to be here It is course unnecessary to start taking microscopical specimens while carbon is still visible macroscopically in for example the vessels of the ear

SUMMARY

In mice given colloidal carbon intravenously the activity of the reticulo endothelial system can be measured by recording the time of disappearance of carbon particles from dried mounted blood drops A magnification of 128 times was found to suffice The method is simple and compares favourably to spectrophotometric estimation

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THE ULTRASTRUCTURE OF HUMAN AND RAT PERICARDIUM

I. *Parietal and Visceral Mesothelium*

By

TROND KLUGE and TORSTEIN HOVIG

Received 11.vi.67

The ultrastructure of human pericardial mesothelium does not seem to have been studied previously.

Staubesand & Schmidt (22) have examined the pericardial lining cells in rats and cats. Otherwise, studies on the ultrastructure of mesothelium have concentrated on the peritoneum in different animal species (9, 11, 14), so have investigations on absorption from body cavities and on regeneration of mesothelium (8, 10, 14).

Several disagreements exist concerning the structure and functional properties of mesothelium. Observations on cytoplasmic structures, cell borders, cell junctions and basement membrane differ. In studies on absorption from body cavities (2, 10, 11) opinions seem particularly variable.

The present work was undertaken with the following purpose:

1. To study the ultrastructure of human and rat pericardium.
2. To make a comparison between the visceral and parietal surfaces.

In addition, examination of cellular and subcellular anatomy was considered essential as a basis for subsequent studies on absorption from the pericardial cavity.

MATERIALS AND METHODS

Adult male and female rats with average weight 300 g were anaesthetized with ether alcohol ([illegible] 1) and the heart was opened by a left or bilateral thoracotomy. Care was taken to avoid blood contamination. Fixative was applied to the outer surface of the exposed pericardium for 30 sec. A small needle was introduced into the pericardial cavity and 0.5 cc of fixative injected. The pericardial sac was opened through a vertical incision and more fixative applied to both surfaces for 1 minute. Within this period heart arrest usually occurred.

Thin slices were cut out from both surfaces, marked with sutures to facilitate orientation and immersed in the fixative. Specimens were taken from corresponding areas overlying the right atrium, right ventricle and left ventricle.

This procedure was carried out in a total of [illegible] animals.

Human material Specimens from both pericardial surfaces were obtained in the operating theatre from 5 male and female patients between 18 and 34 years of age. Their diagnoses were Atrial septal defect (2) ventricular septal defect (2) and Tetralogy of Fallot (1). None of the patients had previous or present disease with known influence on pericardial structures. Increased amounts of pericardial fluid were not observed.

Immediately following incision of the parietal pericardium small pieces were removed and immersed in the fixative. Specimens from the visceral surface were taken from the resected portion of the auricular appendage.

Processing Trimming of specimens was performed with care not to injure the surface tissue. Slices were made 20 mm long and 3–4 mm wide with a thickness up to 3 mm. Fixation was carried out in 2 ways

a) 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 hours followed by 1 per cent osmium tetroxide in 0.1 M phosphate buffer (pH 7.1) for 1 hour
b) Osmium tetroxide only with a fixation period of 2 hours

Specimens were kept at 4 C for the whole fixation period. Following dehydration in graded acetones or ethanol they were embedded in Vestopal W or Epon 812.

The specimens were oriented in the blocks with the long axis vertically to permit sectioning vertical to the surface. Correct orientation was secured by light microscopy of thin sections (0.5–1 μ) cut on a Huxley microtome and stained with alkaline toluidine blue. Ultrathin sections were cut on LKB or Huxley microtomes and stained with 1 per cent uranyl acetate in 30 per cent methanol followed by lead citrate (18). They were examined in a Siemens Elmiskop I. Micrographs were taken at initial magnifications of 2 000 to 20 000.

RESULTS

No principal differences were demonstrated between human and rat pericardial mesothelium. Accordingly the ultrastructure of pericardium from the two species will be presented together.

A Parietal Mesothelium

Electron microscopy of the parietal mesothelium revealed a single layer of large flat cells with maximum sagittal diameter (depth) in the area of the nucleus (Fig. 1). The peripheral portions of the cells were often extremely thin. The cytoplasm appeared as a loose framework with numerous pinocytic vesicles and vacuoles (Fig. 1 and 7). These cells will in the following be referred to as "vacuole containing" cells.

Surface Microvilli The cell surface appeared as a triple layered membrane with a diameter of 80–100 Å (Fig. 3 b). Microvilli were projecting into the pericardial cavity (Fig. 1 and 4). In most cells they were scarce or lacking in the area above the nucleus. Most appeared as simple cylindrical structures while a few showed fork like divisions close to their attachment. The latter type seemed to occur mainly in the vicinity of cell junctions (Fig. 4 c). The length of microvilli was up to 3 micron and the diameter was approximately 800–1000 Å. In cross sections they appeared round or ovoid bordered by the plasma membrane and with a less electron dense interior. In longitudinal sections microfilaments were observed (Fig. 5).

Vesicles and vacuoles Numerous pinocytic vesicles were scattered in the cytoplasm. Their diameter was fairly constant from 400 to 600 Å

They were particularly numerous along the cell surface sometimes in communication with the pericardial cavity (Figs 1 and 4 b) All stages were represented from impressions of the surface membrane up to complete vesicle formation (Fig 7 a) Along the basal borders of the cytoplasm vesicles appeared in continuity with the space between the plasma membrane and the basement membrane (Figs 7 and 8)

Large vacuoles were also present in the cytoplasm Their bordering membranes appeared finely granular and the lumina contained varying amounts of amorphous material (Figs 7 and 10) Some of the vesicles seemed to fuse and form vacuoles (Fig 10) Vacuoles also occurred close to or communicating with the intercellular spaces (Fig 3)

Endoplasmic reticulum The endoplasmic reticulum was very scanty and could not be demonstrated in all cells When present it usually was concentrated in the vicinity of the nucleus It always seemed to be rough surfaced (Figs 1 and 4) Some free ribosomes were scattered in the cytoplasm (Fig 5)

Mitochondria These were present in varying amounts and appeared ovoid or circular with a double membrane and a system of cristae (Figs 9 and 10) Some displayed a well preserved membrane while their interior seemed irregular and disorganized (Fig 10)

Electron dense bodies Dense bodies were sometimes observed in mesothelial cells of both surfaces They varied considerably in size usually within the range of 0 3 to 0 7 micron They were surrounded by a triple layered single membrane approximately 100 A in diameter Their interior was occupied by a homogenous electron dense substance (Fig 7 c)

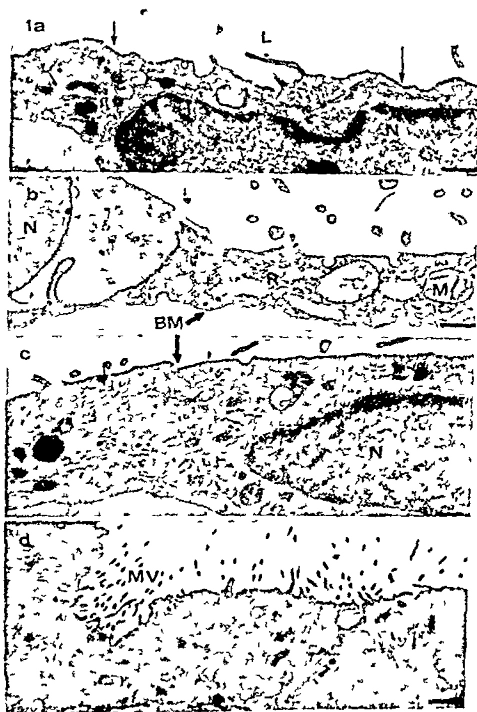
Golgi apparatus Golgi apparatus were detected in some cells in the proximity of the nucleus or more peripherally in the cytoplasm (Fig 11 b)

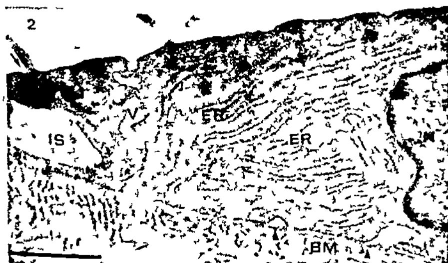
Microtubules Some cells contained structures interpreted as micro tubules with a diameter of approximately 200 A (Fig 11 a) They were present in the deeper layers of the cytoplasm and were organized in a regular pattern with their long axis parallel to the surface

Microfilaments Cytoplasmic filaments were observed in the micro villi (Fig 5 a) and in connection with desmosomes (Fig 5 b)

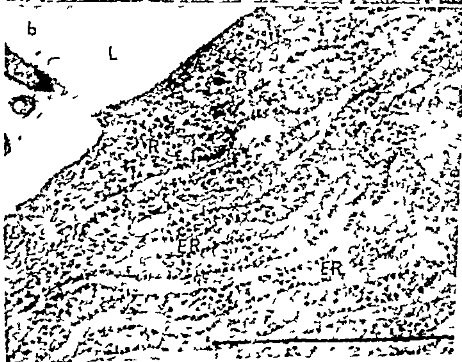
Nucleus Most cells contained a centrally located nucleus without indentations Occasionally two nuclei appeared to be present within one cell (Fig 9) Nucleoli were seldom detected and mitotic figures were not observed

Basement membrane In some sections the distinction of a basement membrane was different even at high magnifications In most areas however all mesothelial cells were seen resting on a basement mem brane which appeared identical in the two species It appeared as a finely granular line with a thickness of approximately 80 200 A (Figs 7 and 8) The membrane seemed smooth and uniform in the majority


1a
L
N
b
N
R
M
BM
c
N
d
MV



3
V
IS
N
BM


6a
L
ER
IS
V
b
L
R
R
ER
ER


7a
PV
L
b
M
BM
DB
DB
DB
V


IIa
N
mt
PV

Legends to Figs 1–11

Fig 1 Typical appearance of pericardial mesothelium The cytoplasm is of low electron density with scattered mitochondria (M) and some ribosomes (R) Microvilli (MV) are projecting into the pericardial lumen (L) Pinocytic vesicles partly communicating with the pericardial cavity are indicated by arrows Nucleus (N) Basement membrane (BM) a Parietal surface homo × 9 000 b Parietal surface rat × 9 000 c Parietal surface homo × 9 000 d Parietal surface rat × 9 000

Fig 2 Micrograph demonstrating an ER cell (see text) The abundant endoplasmic reticulum (ER) occupies the whole cytoplasm from the nucleus (N) to a system of vacuoles (V) To the left is a cell border with a tight junction (arrow) above a wide intercellular space (IS) Triangles indicate structure variations of the plasma membrane above the basement membrane (BM) Visceral surface rat × 24 000

Fig 3 Unusual configuration of visceral mesothelial cell The arrow indicates a tight junction (TJ) above the intercellular space (IS) Cytoplasmic vacuoles (V) Nucleus (N) Visceral surface homo × 15 000

Fig 4 A varying number of microvilli (MV) extend into the pericardial cavity (L) They usually appear as single cylindrical structures (Fig b) but occasionally show fork like divisions mainly in the vicinity of cell junctions (arrow Fig c) The submesothelial area contains bundles of collagen (COL) a Parietal surface rat × 20 000 b Parietal surface homo × 30 000 c Parietal surface rat × 12 000

Fig 5 Surface at higher magnification with triple layered surface membrane (triangles Fig b) Microvilli contain microfilaments (arrows) Confluence of vesicles to a larger vacuole (V) is suggested The cytoplasm contains scattered ribosomes (R) and thin filaments converging upon a desmosome (D) a Parietal surface homo × 30 000 b Parietal surface rat × 60 000

Fig 6 Abundant endoplasmic reticulum (ER) gives the typical appearance of the ER cells (see text) The reticulum is rough surfaced and frequently arranged as parallel tubules Some free ribosomes (R) are present In Fig a an intercellular space (IS) separates the ER-cell from a vacuole-containing cell (V) Pericardial lumen (L) a Visceral surface homo × 30 000 b Visceral surface rat × 60 000

Fig 7 Pinocytic vesicles (PV) vacuoles (V) and dense bodies (DB) In Fig a the vesicles are forming from or communicating with the surface (arrows) All stages are represented from impressions of the surface membrane to completely formed vesicles Fig b demonstrates pinocytic vesicles in communication with the subcellular space (arrows) above the basement membrane (BM) In Fig c dense bodies (DB) of varying size are present in the cytoplasm Lumen (L) Mitochondria (M) a Parietal surface homo × 30 000 b Parietal surface rat × 30 000 c Parietal surface homo × 45 000

Fig 8 Interruptions of the basement membrane (BM) are suspected in certain areas (heavy arrow) Small arrows indicate communications between pinocytic vesicles (PV) and the subcellular space Mitochondria (M) Heavy bundles of collagen (COL) are present beneath the thin [illegible] Lumen (L) a Visceral surface rat × 18 000 b [illegible] surface homo × 24 000

Fig 9 Mesothelial cell apparently with [illegible] (see text) Visceral surface rat, × 6 000

Fig 10 Pinocytic vesicles (PV) seem to [illegible] form larger vacuole (V) Some larger vacuoles are surrounded by [illegible] triple layered membrane and contain electron dense material (arrows) The [illegible] may be related to the dense bodies (see text) The mitochondria (M) display a double membrane and a system of cristae a Parietal surface rat × 30 000 Parietal surface homo × 4 000

Fig 11 Microtubuli (mt) in mesothelial cell The diameter is approximately 200 A and the surface smooth (arrow Fig a) In Fig b a Golgi-complex (G) is present in the cytoplasm Mitochondria (M) Vacuoles (V) Nucleus (N) Basement membrane (BM) a Visceral surface rat × [illegible]000 b Visceral surface homo × 30 000

of sections but interruptions were occasionally suspected (Fig. 8) with vesicles and intercellular spaces appearing to communicate with the subcellular area. Between the basement membrane and the plasma membrane a zone of less electron density was present. This space frequently communicated with cytoplasmic vesicles and vacuoles (Figs. 7 b and 8).

B. *Visceral Mesothelium*

Vacuole containing cells were most frequently present also on the visceral surface. However in both species the visceral mesothelium was irregular and displayed scattered cells of a different appearance (Figs. 2 and 3). Their most characteristic feature was an abundant rough surfaced endoplasmic reticulum frequently arranged as parallel tubules (Fig. 6). Usually it occupied most of the cytoplasm and was well developed also close to the cell borders and junctions (Figs. 2 and 6). These cells possessed few microvilli, vesicles, vacuoles and mitochondria. They will in the following be referred to as ER cells. Occasionally they were lying 2 or 3 together but usually they occurred solitary between cells of the vacuole containing type (Fig. 6 b). They were resting on the common continuous basement membrane. It should be stressed that no intermediate cell type was observed; every cell examined seemed to belong to one or the other of the two entities. *ER cells* were never observed on the parietal surface.

C. *The Subcellular Area*

Beneath the mesothelium and basement membrane was an irregular lacework of tissue with scattered fibroblasts and macrophages. Mast cells and lymphocytes were occasionally observed. The most conspicuous feature was an abundance of collagen frequently arranged in heavy bundles (Fig. 8). On the visceral side the submesothelial tissue extended down between the fibres of the myocardium. Capillaries were extremely scarce in the submesothelial tissue of both surfaces.

DISCUSSION

In the present study the ultrastructure of human and rat pericardial mesothelium appeared identical. Comparative studies in rats and cats have also shown striking similarities (22).

The present investigation demonstrates that two different cell types, i.e. vacuole containing cells and ER cells, are present in pericardial mesothelium. Previous work on human synovial membrane which has many properties in common with mesothelium also revealed 2 main cell types (4). One type was commonly present and contained numerous vacuoles and filopodial projections while the other was less frequent and characterized by an abundant endoplasmic reticulum and

absence of vacuoles In a study on rabbit synovia *Ball* (2) observed rapid uptake of iron particles in the vacuole containing cells while ER-containing cells rarely absorbed tracer substance

The endoplasmic reticulum of the ER cells observed in the present study seemed to occupy most of the cytoplasm (Fig 2) and was definitely rough surfaced (Figs 4 and 6) Previous works on pericardial (22) and peritoneal (3 8) mesothelium led to the conclusion that the reticulum is very scanty and usually located in the proximity of the nucleus Some authors (3 22) describe the reticulum to be sometimes granular while *Eskeland* (8) and *Fukata* (11) state that the endoplasmic reticulum is of the smooth surfaced variety This disparity from observations made in the present study cannot be explained by differences in technique since most sections were prepared by the same method as those employed by *Eskeland* (8)

An important question is whether the described ER cells really are mesothelial cells *Eskeland* (8) put forward the following criteria for identification of true mesothelium

1 Microvilli of a characteristic appearance on the cell surface
2 Tight junctions between adjacent cells
3 Cells resting on a basement membrane
4 Pinocytic vesicles in the cytoplasm

In the present study the first 3 criteria were all fulfilled in the ER cells However instead of pinocytic vesicles the cytoplasm of these cells was occupied by the abundant endoplasmic reticulum

Other cell types are known to be occasionally present between mesothelial cells The only possibility of confusion however is confined to macrophages (8) or Wanderzellen (22) These cells never rest on a basement membrane their nuclei show typical indentations and often nucleoli they possess numerous mitochondria and phagocytic vacuoles and their cytoplasmic processes are irregular All of these characteristics are different from those observed in the ER cells Accordingly it is strongly suggested both positively and by exclusion that the ER cells are of true mesothelial character

The next question is whether they represent a special type of normal mesothelial cells a particular state of function or simply are growing cells in an earlier stage of differentiation

A well developed ER and numerous free ribosomes are observed in growing endothelium (6 21 24) and during the healing of peritoneal wounds (8) Furthermore numerous ribosomes are commonly observed in undifferentiated cells (15) and few cytoplasmic vesicles is also considered a sign of low differentiation (21)

Hence one might assume that the ER cells represent growing mesothelium which has not reached its full state of differentiation A similar picture might be displayed by mesothelial cells in a particular state of

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THE ULTRASTRUCTURE OF HUMAN AND RAT PERICARDIUM

2 *Intercellular Spaces and Junctions*

By

TROND KLUGE and TORSTEIN HOVIG

Received 18 vi 67

In previous studies on mesothelium (3 7 10) considerable variations in the course and configuration of cell borders and intercellular spaces are reported As for the pericardial mesothelium (10) some confusion seems to be present with regard to the distinction between cytoplasmic vacuoles and intercellular spaces

Tight junctions are considered to be commonly present between mesothelial cells and some authors suggest that they encircle every cell as continuous structures (6 13) Most authors agree that desmosomes on the other hand are rarely detected in mesothelium However they are frequently encountered in various epithelia (4)

As stressed in previous works on peritoneal mesothelium (2 3) the *en face* inspection of mesothelium is of great value in light microscopy particularly in the evaluation of cell borders It was felt that a similar arrangement should be tried for electron microscopy

The purpose of the present work was to examine cell junctions and intercellular spaces in horizontal as well as in vertical sections

MATERIALS AND METHODS

Material for electron microscopy as [illegible] from 5 surgical patients and 22 adult male and female rats The [illegible] ed according to methods described in a previous publication (7) However [illegible] rtant addition as made to the procedure Specimens of parietal pe[illegible] oriented and trimmed in such a fashion that ultrathin sections could be m[illegible] parallel to the mesothelial surface However technically difficult this procedure [illegible] eatly facilitated by the presence of numerous microvilli on the cell surfaces When nothing but cross sections of microvilli were present in the first series of thin sections it was highly probable that the following sections would be almost parallel to the surface

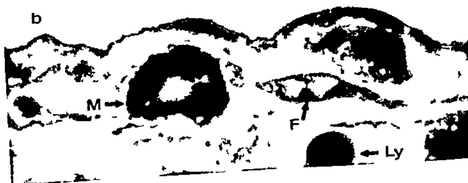
Due to the variable and uneven surface of the visceral pericardium the method could be applied only to the parietal surface

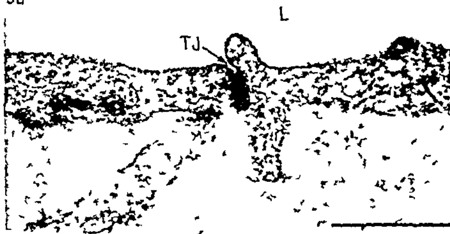
Fixation was performed with glutaraldehyde followed by osmium tetroxide (3 7) or with osmium tetroxide only Embedding media were Vestopal W and Epon 812 Ultrathin sections were cut on LBK and Huxley microtomes and examined in a Siemens Elmiskop I

Thin sections for light microscopy (0 5–1 μ) were cut on a Huxley microtome and

2a

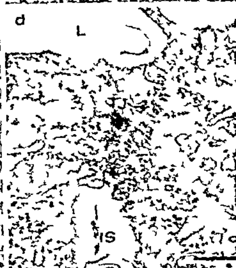


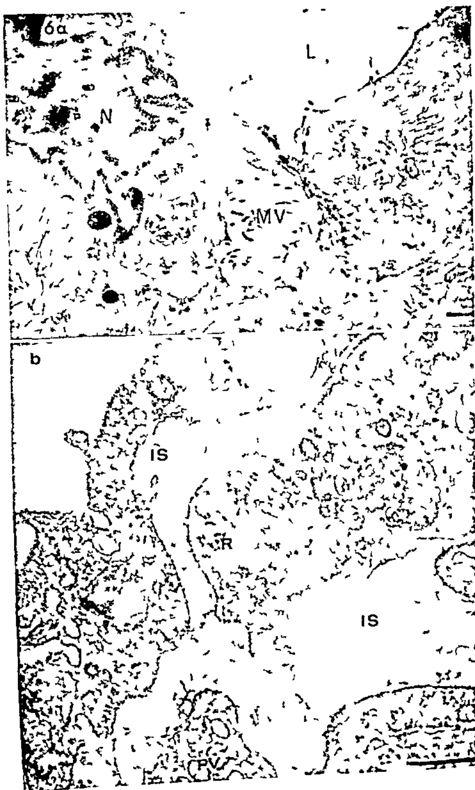



3a
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TJ


b
BM


c
N


d
L
IS


6a
L
N
MV
b
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R
IS
PV

7a

L

L

IS

N

BM

b

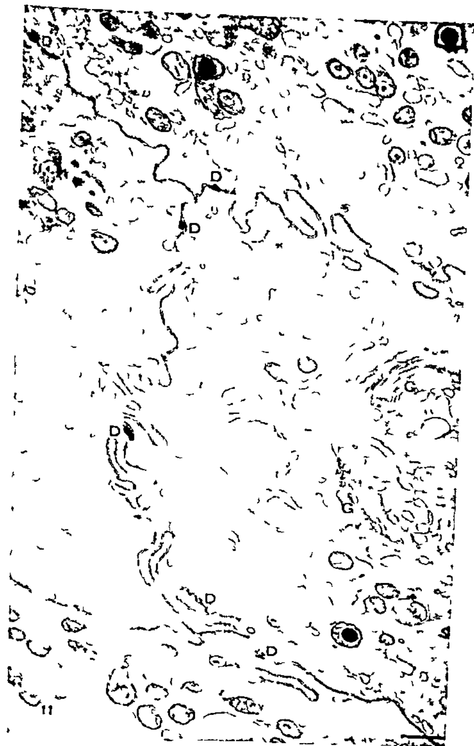
TJ

D

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BM


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11

Legends to Figs 1–12

Fig 1 Light micrograph of surface preparation Each cell appears as a single entity (see text) Cell borders outlined by impregnation with silver nitrate Haematoxylin staining Visceral surface rat × 1 000

Fig 2 Light micrographs of vertical sections Microvilli (MV) are visible on cell surface Wide intercellular spaces or large cytoplasmic vacuoles are indicated by arrows (Fig a) Mast cells (M) fibroblasts (F) and some few lymphocytes (Ly) are present in submesothelial tissue Toluidine blue staining a Parietal surface rat × 1 000 b Visceral surface rat × 1 000

Fig 3 Cell junctions (CJ) and intercellular spaces (IS) are extremely variable Some are short and narrow (Fig a) occupied by a tight junction (TJ) Others are long and narrow (Figs b and c) and many spaces appear to be continuous with the subcellular space (triangle Fig b) Lumen (L) Nucleus (N) a Parietal surface rat × 30 000 b Parietal surface rat × 18 000 c Visceral surface homo × 12 000 d Visceral surface homo × 12 000

Fig 4 Cell junctions with a long and complicated course with neighbouring cells overlapping each other (Fig a) Wide intercellular spaces (IS) appear to communicate with the subcellular space (triangles Fig b) Tight junction (TJ) Microvilli (MV) a Visceral surface homo × 12 000 b Visceral surface rat × 12 000

Fig 5 Different appearance of intercellular spaces Some appear connected with systems of vacuoles (V) and communicate with the subcellular space above the basement membrane (BM) Cytoplasmic processes (CP) project into wide intercellular spaces (IS) In Figs a and c interruptions of the basement membrane (BM) are suspected (triangles) Lumen (L) Microvilli (MV) a Parietal surface homo × 17 000 b Parietal surface rat × 16 000 c Parietal surface homo × 48 000

Fig 6 Microvilli (MV) are projecting into intercellular spaces (Fig a) In Fig b wide intercellular spaces (IS) are present between 3 mesothelial cells Pinocytic vesicles (PV) and ribosomes (R) are scattered in the cytoplasm a Visceral surface homo × 9 000 b Parietal surface rat × 18 000

Fig 7 Wide intercellular spaces (IS) In Fig b a desmosome (D) with converging filaments (arrow) is present below a tight junction (TJ) The triangles indicate connection between the intercellular (IS) and subcellular space above the basement membrane (BM) a Visceral surface rat × 12 000 b Visceral surface homo × 20 000

Fig 8 Arrow indicates widening of an intercellular space (IS) below a tight junction (TJ) Cytoplasmic pro es es (CP) are extending into the space Visceral surface rat × 24 000

Fig 9 Tight junctions are not al s demonstrable between vertically sectioned cells (see text) Intercellula space (IS) Mitochondria (M) Nucleus (N) Visceral surface rat × 24 000

Fig 10 Section oriented parallel t the surface Compl x configuration of cell borders with variable spaces (IS) and intertwin ng of cytopla mic processes Vesicles and vacuoles (V) Nucle (N) Mitochondr a (M) Glut raldehyde and o mium tetroxide fixation P i tal surface at × 6 000

Fig 11 Horizontal section Se eral desmosome (D) are present along the cell borders Golgi appar tu (G) appear in the cytoplasm Osmium tetroxide fixation Pariet l su face rat × 12 000

Fig 12 Cells of different rigin in mesothelium In Fig a a lymphocyte (Ly) is present in the interc llular pace (IS) In Fig b a h t oc te (H) with typical indentat ons of the nu leus appears between mesothelial cells (MES) Lumen (L) Va u le (V) M tochondria (M) a Parietal surface homo × 18 000 b Par tal suface rat × 18 000

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ACQUIRED IMMUNITY AFTER SURGICAL REMOVAL OF SUBCUTANEOUS EHRLICH'S ASCITES CARCINOMA

By

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The observation that both spontaneous regression and surgical removal of transplanted tumours may produce a state of immunity to a second transplant was made more than 50 years ago (review by Woglom 1913). Such experiments were only successful when homotransplanted tumours were used. They lead to the converse generalization as expressed by, among others, Haaland (1909), that it was seemingly impossible to immunize an animal against its own tumour cells.

Established lines of homotransplantable tumours grow progressively like isogeneic transplanted or spontaneous tumours and are not rejected in spite of theoretical incompatibility. Their rapid growth makes *a satisfactory excision difficult, but implantation of such tumours into* the tail of mice followed by amputation as recommended by *Andervont* (1932) provides a simple immunizing procedure.

The purpose of the present experiments was to use this method to study the effect of increased host resistance on the growth of Ehrlich's ascites carcinoma, and to test the truth of the indications that subcutaneous transplants of EAC may be more sensitive to the host immune response than intraperitoneal transplants (*Hartveit* 196[illegible]; *Thunold* 1967a).

The following experiments were carried out.

TUMOUR GROWTH AND SURVIVAL TIME AFTER SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF EHRLICH'S ASCITES CARCINOMA IN IMMUNIZED MICE

Material and Methods

Mice. These were males (weight 28 to 30 g) and females (weight 24 to 26 g) of similar age from the closed colony kept at this Institute (*Thunold* 1966a).

Tumour. The Ehrlich ascites carcinoma used in these experiments was from 10-day-old transplants in female mice. All animals were given 0.05 ml of a washed suspension of tumour cells in saline, containing 4.0 to 4.5 × 10^{4} cells.

Experimental Procedure

Primary Tail Transplants

These were obtained by injecting EAC subcutaneously midway up the tail. Care was taken not to inject intravascularily. Amputation according to Andervont's method was carried out after 7 days in one group and 14 days in another: the animal was etherized and the tail with the tumour cut with a sharp knife about 10 mm from the root. The stump was painted with tincture of iodine to prevent infection. Histology confirmed the presence of tumour in the amputated tails. The stumps healed completely within 2 weeks and there were no deaths.

Mice with visible tumour recurrence were excluded and preliminary studies had shown that metastases were absent at 7 days of tumour growth and only occasionally present at 14 days.

Secondary Transplants

Groups of 12 male and 12 female mice that had had primary transplants removed after 7 (gr. I) or 14 (gr. II) days growth were reinjected 14 days after with EAC either subcutaneously or intraperitoneally. Non-immunized mice received primary subcutaneous or intraperitoneal transplants after having had similar amputation of their tails (gr. III).

Subcutaneous growth. The mice were inspected daily for tumour growth. The day of visible tumour appearance was recorded, and the tumour size was calculated by measuring three diameters at right angles by callipers. From these values the individual and the group mean diameters were calculated.

Intraperitoneal growth. These mice were also inspected daily after tumour inoculation and the weight recorded to the nearest 0.5 g. At death the survival time was recorded in days. The ascitic fluid was removed and measured and the total packed cell volume (PCV) and the cell free fluid volume determined as previously (*Thunold* 1966a).

Tertiary Transplants

Mice surviving primary and secondary transplants were reinjected intraperitoneally with EAC.

Results

Subcutaneous Growth

The *number of mice developing tumours* in each group is shown in Fig. 1. After a short period in which no tumours could be seen tumours appeared in all the control mice with primary transplants. The *latent period* (in days) of secondary transplanted tumours was significantly prolonged (gr. III ♂ (5.5 ± 1.9) *vs* gr. I ♂ (12.3 ± 8.1) and gr. II ♂ (18.0 ± 7.6): $P < 0.05$; gr. III ♀ (6.4 ± 2.4) *vs* gr. I ♀ (13.0 ± 11.5) and gr. II ♀ (22.5 ± 6.8): $P < 0.05$). There was no sex difference within the groups, neither was there any difference between groups I and II.

Two of the male mice and 6 of the female mice in which primary tumours had been removed at 7 days remained tumour free. Four of the males and 8 of the females which had had primary transplants removed at 14 days remained tumour free. These mice proved resistant to a tertiary transplant also. The difference in number of mice developing tumours in these groups compared to the control groups with primary transplants was significant for both sexes (gr. III *vs* I: ♂ $P < 0.05$, ♀ $P < 0.005$; gr. III *vs* II: ♂ and ♀ $P < 0.005$). The sex difference

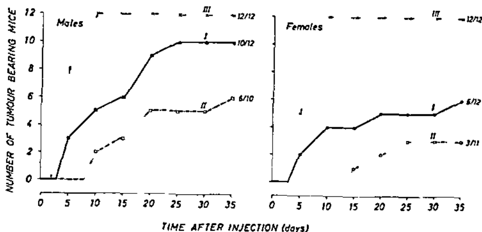


Fig 1

Subcutaneous growth of Ehrlich s ascites carcinoma The curves give the time between tumour injection and tumour appearance Each point represents the number of mice with tumour at various times after tumour injection The numbers at the end of each curve represent mice with tumour related to the total number inoculated

7 days primary growth	gr I ♂	■——■	gr I ♀	●——●
14 days primary growth	*gr II* ♂	□- - -□	gr II ♀	○- - -○
Controls	gr III ♂	×----×	gr III ♀	×- ×

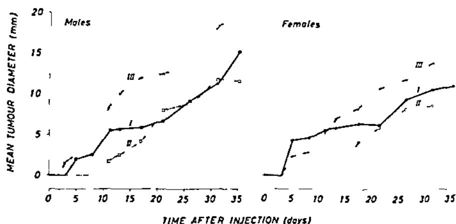


Fig 2

Subcutaneous growth of Ehrlich s ascites carcinoma Mean diameters are plotted for mice developing tumour in each group For explanation of symbols see text to Fig 1

within the groups was not significant neither was the difference between groups I and II

The *mean tumour diameter* in the tumour bearing mice of all groups is shown in Fig 2 Once the tumour appeared the slope of the growth curves was similar in all three groups showing that the growth rate was the same As the tumours ulcerated at from 4 to 6 weeks after transplantation further measurements and survival time registration were considered unwarranted *All mice that developed tumours died*

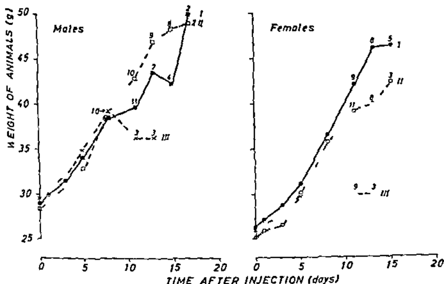


Fig 3
The mean increase in body weight after intraperitoneal injection of Ehrlich s ascites carcinoma The numbers represent surviving animals For explanation of symbols see text to Fig 1

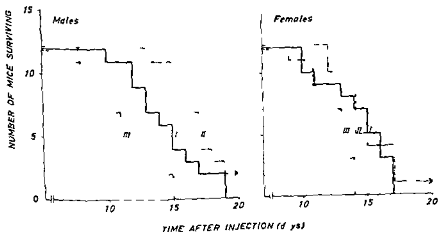


Fig 4
The number of mice urviving at various times afte intraperitoneal injection of Ehrlich s as ites car inoma For explanation of symbols see text to Fig 1

Intraperitoneal Grou th

Body weight A rapid increase in body weight (see Fig 3) occured at first in all groups But about 8 days after transplantation the increase in body weight stopped in the control groups while it continued in

TABLE I

The Correlation between Packed Cell Volume (PCV) and Cell Free Fluid Volume and the Survival Time in Male and Female Immunized and Control Mice Injected Intraperitoneally with Ehrlich's Ascites Carcinoma

Groups	Pretreatment	No and sex of animals	PCV Coefficient of correlation (r)	P	Fluid Volume Coefficient of correlation (r)	P
I	EAC (7 days)	12 ♂	−0.7699	<0.01	+0.7973	<0.01
		12 ♀	−0.8593	<0.001	+0.8489	<0.001
II	EAC (14 days)	10 ♂	−0.7238	<0.02	+0.7637	<0.02
		11 ♀§	−0.8580	<0.001	+0.8738	<0.001
III	Nil	12 ♂	+0.3779	>0.20	+0.4996	>0.10
		12 ♀	+0.1997	>0.50	+0.4030	>0.10

Two survivors not included   § One survivor not included

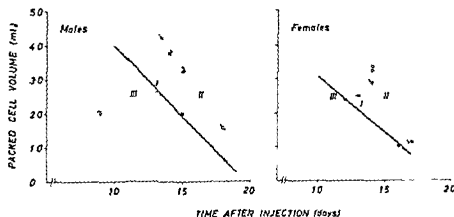


Fig 5a

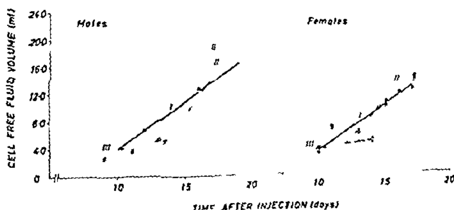


Fig 5b

The scatter diagram and the regression lines for the relationship between the total volumes of packed cells and cell free fluid and the time of death after intraperitoneal injection of Ehrlich's ascites carcinoma For explanation of symbols see text to Fig 1

those groups with secondary transplants These differences in weight between the groups were less marked in male than in female mice

Survival time As Fig. 4 shows the survival time (in days) of the mice with secondary transplants was greater than that of the controls (gr III ♂ (10.9 ± 2.6) *vs* gr I ♂ (14.6 ± 2.8) and gr II ♂ (16.4 ± 1.6) $P < 0.01$ and $P < 0.001$ gr III ♀ (12.1 ± 1.5) *vs* gr I ♀ (14.3 ± 2.7) and gr II ♀ (14.5 ± 1.9) $P < 0.05$ and $P < 0.01$) No significant sex difference appeared and there was no difference between groups I and II Three mice (two males and 1 female) in which the primary tumour had been amputated after 14 days growth survived the secondary transplant These mice were resistant to a tertiary transplant also They were excluded from statistical analysis

Total volumes of packed cells and cell free fluid The relationship between these values and the time of death is shown in Table 1 There was a significant negative correlation between the packed cell volume and the survival time and a significant positive correlation between the fluid volume and survival time in all groups with secondary transplants Thus there was a lower cell volume and more fluid in late survivors compared to those dying early In the control groups with primary intraperitoneal transplants no such relationship was seen The scatter diagram and the regression lines for all groups are shown in Figs 5a and 5b It can be seen from these that most of the late survivors in the immunized groups have a lower packed cell volume and higher fluid volume than the controls

Total volume of packed erythrocytes There was a negative correlation between the blood content and the time of death in all groups with secondary transplants and in the controls but this correlation was not statistically significant Any difference between the mean values of the blood content in animals in the various groups could not be demonstrated either

INTRAPERITONEAL GROWTH AND CYTOLOGY OF EHRLICH'S ASCITES CARCINOMA IN IMMUNIZED MICE

Analysis of the results in the previous experiment showed a lower cell volume in the late survivors with secondary intraperitoneal transplants as compared with those dying early These measurements include tumour cells and a varying admixture of host cells and it became clear that cytological studies were needed before a further interpretation of the changes in tumour growth could be attempted

Material and Methods

Mice and tumour Females only of the closed colony weighing between [illegible]3 and [illegible] g were used The Ehrlich ascites carcinoma used at each transplant contained the same number of cells as in the previous experiment

The results from the secondary intraperitoneal transplants showed that protection was less effective as only 3 out of 18 mice survived. The results of the analysis of the tumour ascites in the survival time study showed however that late survivors in the immunized groups had a lower packed cell volume and more fluid, i.e. inflammatory exudate, than those dying earlier. In contrast to this, no such relationship with time could be demonstrated in the mice with primary intraperitoneal transplants. The latter was in keeping with earlier findings in previously untreated mice with intraperitoneal EAC (*Thunold* 1966b). The cytological investigation showed reduced amounts of tumour cells and more fluid in the immunized mice than in the controls. This group difference was pronounced in the late survivors. Such immunized mice as had been investigated earlier had greater amounts of tumour cells in the ascitic fluid. It should be emphasized, however, that the latter showed great individual variations in the tumour values. This was expected as these mice include both early and late survivors. In contrast the mice investigated later will consist mainly of individuals with a greater degree of protection, as those dying early from tumour growth are not included.

These findings showed that tumour cell growth was inhibited in the immunized mice. In addition, inflammatory reactions had also been stimulated. The treatment of the host thus seemed to have contrary effects on the outcome of tumour growth: protection against the tumour had been built up in some mice, but this was accompanied only by an increase in survival time and not by survival, as a progressive accumulation of an inflammatory exudate lead to the death of the animals. *In vivo* experiments by *Apffel et al.* (1966) indicated that the presence of cell free ascitic fluid protected tumour cells from the host response in immunized mice. The demonstration by *Hartveit* (1966) that mouse gamma globulin inhibited lysis of tumour cells *in vitro* might also be pertinent to the present findings.

The experiments showed that a transient effect of immune reactions (*Thunold* 1967 a, b) could be made effective by previous injection of living tumour cells and removal of the primary transplant. These findings were in general agreement with previous observations that effective immunity can be induced against EAC after pretreatment wit tumour cells killed or damaged by irradiation (*Révész* 19.., *Donak* & *Mitchell* 1959, *Ma urel* & *Duplan* 1959, 1961, *Mckee et al.* . by repeated withdrawal of living tumour ascites (*Apffel et al* Such immunity was found to follow the general rules appl transplantation immunity against homografts as it could be , by preceding whole body irradiation (*Ba m* & *Duplan* 1963) also be transferred by lymphoid cells from immune mice normal lymphocytes (*Ma urel* & *Duplan* 1961). The quot the results of the present experiments indicate that antigen(s) not present in normal mice of different stra

mour arose in a mouse of unknown genetic origin the question of cancer specific antigens cannot be approached

Even though previous works have indicated that immunity against a primary transplant of EAC was greater in female than in male mice (*Thunold* 1967 *a b*) sex differences in induced immunity against a secondary transplant of the same tumour could not be demonstrated with certainty The present experiment further showed that the immunity against a subsequent graft of EAC was as effective after 7 as after 14 days of primary tumour growth and it confirmed the previous observation that it was easier to demonstrate immunological inhibition of subcutaneous than of intraperitoneal transplants of EAC

SUMMARY

The effect of surgical removal of a primary tail transplant of Ehrlich s ascites carcinoma on the growth of secondary subcutaneous or intraperitoneal transplants was studied

The secondary subcutaneous tumours showed a longer latency period and a reduced number of takes as compared with controls Contrary to this the secondary intraperitoneal tumours grew in most mice Inhibition of tumour growth was achieved This was accompanied not by survival but only by increase in survival time as progressive accumulation of inflammatory exudate lead to the death of the animals

These results indicated a contrary effect of the immunizing procedure inhibition of tumour cell proliferation and strengthening of the inflammatory reactions The previous observation that it was easier to demonstrate immunological inhibition of subcutaneous than of intraperitoneal transplants of EAC was confirmed

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ULTRASTRUCTURAL INVESTIGATIONS ON THE CELLULAR MORPHOGENESIS OF EXPERIMENTAL MOUSE AMYLOIDOSIS

By

POUL RANLØV and JOHN WANSTRUP

Received 12.vi.67

The intimate morphologic relationship between newly formed amyloid and reticuloendothelial cells, especially the so-called "PAS-positive cells" described by *Teilum* (1956), was considered highly suggestive of a cellular morphogenesis of this substance. However, further investigations employing the light microscope have all proved unsuccessful in demonstrating a possible intracellular localization of the amyloid substance.

After the demonstration of the characteristic fibrillar ultrastructure of amyloid (*Cohen & Calkins* 1959) the need for further investigations of the experimentally induced amyloidosis employing the electron microscope became obvious. Furthermore, these investigations were facilitated by the appearance of improved techniques with regard to the procedures of fixation and embedding of the specimens.

The occurrence of moderately developed ergastoplasm in cells localized in the immediate vicinity of amyloid deposits was described by *Caesar* (1960), although coalescence of ergastoplasmic cisterns with extracellular amyloid in certain areas could be demonstrated, this author concluded that truly intracellular amyloid did not occur in his material. The interpretations of his results were largely identical to those of results later reached by *Cohen & Calkins* (1960) and by *Cohen et al.* (1960) employing an experimental model of casein-treated rabbits. Based on ultrastructural investigations of casein-induced mouse amyloidosis *Hjort & Christensen* (1961) also described the development of ergastoplasm in neighbouring cells, but contrary to Caesar's findings these authors stressed that this ergastoplasm appeared much less differentiated and it contained fewer ribosomes than is otherwise usual in plasma cells. *Hjort & Christensen* further described the occurrence of a fibrillar substance, probably amyloid, within the cytoplasm of

This investigation was supported by a grant from *Ingeniør of Frederikssund Søren Alfred Andersen's Legat*.



Fig 1
Paraffin embedded section of spleen from mouse showing amyloidosis (degree 3 4) after 24 daily injections of casein Haematoxylin eosin × 140

these cells but due to a less suitable technique (embedding in a metacrylate medium) it seems difficult from their published photographs to ascertain whether this substance actually did form an integrating part of the cytoplasm or whether it was separated from it by membrane like structures

This difficulty in distinguishing between truly intracellular amyloid and invaginated extracellularly located amyloid material seems particularly pronounced in the liver In this organ the presence of numerous interdigitations between cell borders rather tends to add to the confusion than to facilitate such a distinction These difficulties seem obvious from the investigations on experimental liver amyloidosis in the mouse reported by *Sorenson et al* (1964)

Cohen et al (1965) reported the maintenance for weeks of amyloid

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Fig

Perifollicular cellular from amyloid mouse spleen Reticular cells in various stages of differentiation in close relation to and sometimes confluent with abundant fibrillar amyloid substance are seen Most cells appear rather primitive their cytoplasm being dominated by free ribosomes forming clusters Other cells however show advanced differentiation—i e a prominent [illegible] several mitochondria [illegible] complex vesicular structures (black arrows) and fine spherical membrane [illegible] (white arrows)

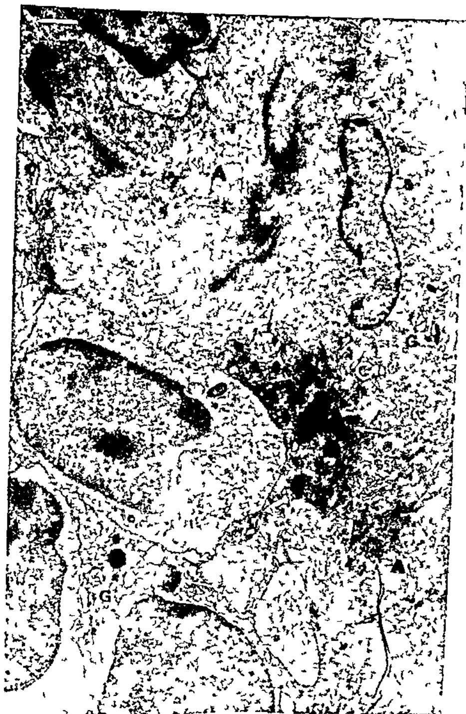


Fig 2



Fig 3

in tissue cultures of rabbit spleen explants Electron microscopic examinations revealed small amounts of a fibrillar material within the cytoplasm of some thus cultured reticuloendothelial cells In view of the less physiological conditions of their *in vitro* experiment it might be argued that the occurrence of a few amyloid like fibrils does not fully justify their identification as intracellularly formed amyloid substance It remains however a possibility

In the following an account is given of some observations on the ultrastructure of experimental mouse amyloidosis The results indicate apart from the demonstration of numerous cells with intracellularly located amyloid a certain sequence of degenerative changes in reticular cells apparently related to a shift from an extracellular to an intracellular precipitation of amyloid fibrils in such cells

MATERIAL AND METHODS

The material spleen and livers was derived from adult (3–4 months old) female C3H mice Prior to killing each animal received a total of 24 daily subcutaneous injections of ½ ml of a 5 per cent casein solution in 0 5 per cent NaOH the solution was sterilized On the day after the last casein injection spleens and liver tissues were removed under ether anaesthesia Small pieces of the e tissues were immediately after removal placed in glutaraldehyde 6 per cent followed by postfixation in OsO_4 2 per cent Embedding medium was Vestopal W For the purpose of orientation semi fine sections were made one micron thi l and stained with toluidine blue Ultrathin sections were cut on an LBI ultramicrotome They were afterstained with uranyl acetate (5 per cent aqueous solution) and/or lead hydroxide a m Reynolds and examine l in a Siemens Elmisl op I Conventional paraffin embedded tissue was stained with haematoxylin eosin with alkal ne Congo red and with the periodic acid Schiff technique Amyloid was identified by its morphology and by its green birefringence with Congo red u der crossed polars

RESULTS

Ordinary light microscopy revealed prominent amyloid formation in all spleens averaging a degree of 3–4 as estimated by the semiquantitative method described by *Christensen & Hjort* (1959) The amount of spleen amyloid appears from Fig 1 The amyloid substance exhibited the usual staining properties and it was constantly seen in close relation to a variety of reticular cells in different stages of differentiation including many basophilic and PAS positive types From a patho and morphogenetical point of view the cellular events in these proliferating perifollicular collars are of special interest and consequently these areas were the main subject of our ultrastructural investigations

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Fig 3

Primit ve ret ul ll fr m amyloi l sp l en closely rel ted to su rounding amyloid substance (A) Th s substance appe rs clearly f brillar and haracterist call ar ranged in right angl to the c ll b le which appears intact The nucleus (N) is large and m lium hromatine rich Apart from a p ominent es ular Golg a ea (G) the cytoplasm seems poorly differentiated



Fig. 5

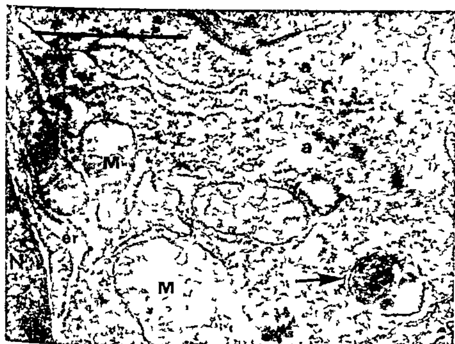


Fig 6

Well differentiated reticular cell from amyloid mouse spleen Frgastoplasmic reticulum (er) many f xed and free ribosomes and a number of swollen mitochondria (M) are seen I resent in the cytoplasm are areas of fibrillar non membrane lined material (a) comparable in stru ture to the extracellular amyloid dep sits (A) Further dense and complex bodies of prcl ably mitochon lrial origin (arrow) are seen The cell borders are intact

larly differentiated cells containing amyloid fibrils (Fig 6) Apart from the areas with integrating fibrillar substance the cytoplasm is dominated by a rough erg stoplasm by swollen mitochondria and by membrane lined dense and complex bodies some of which are probably mitochondrial in origin The cell border however appears as an unbroken barrier between extracellular and intracellular amyloid This is not the case in the cell illustrated in Fig 7 Here too abundant intra and extracellul ir amyloid fibrils are evident but occasionally the cell border appears blurred with a tendency of the cytoplasmic amyloid to continue into the extracellularly located deposits Further a large vesi

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F g

Section of reti ular cell from amyloi l mou e spleen It is adjacent to similar cell and the pl smolemma (arr) apj ear vell l m rl ate l There i abundant erga to plasm (e) an l cl sters of free r b ome C n pieu us is a l rge toplasmic area containing a fil rillar non membran l ne l an l i l l ke sub tance (a) which is exten ling cl se to the n l u (N)



Fig 8

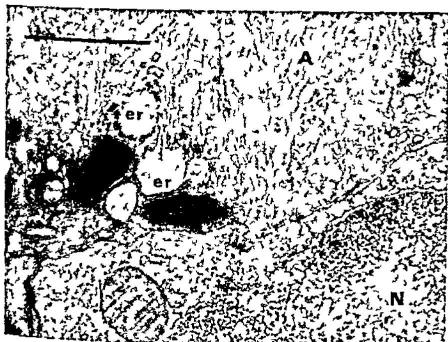


Fig 9

Section from amyloidotic mouse spleen A lymphoc te nucleus (N) is seen A frag ment of the c toplasm of an other cell in continuit with abundant amyl id substance (A) is illustrated The plasmolemma is lost and dilated erga toplasmic saccules are present, sometimes with broken membranes an l thu in continuity with bundles of amyloid fibrils In ad lition dense lyso ome like bodies and complex vesicular structure are seen

DISCUSSION

The possible explanation of the results of the present investigations rests on an issumed existence of two mechanisms of amyloid precipi lation from a preamyloid subst ince produced in splenic reticular cells

One possibility is the secretion of a preamyloid substance which in the extracellular environment is caused to precipitate as bundles of amyloid fibrils arranged in parallel arrays as shown in Fig 3 The other possibility is the prem iture precipitation of fibrils within these

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Fig 8

Reticular cell from perifollicular colla in am loid mouse spleen (conf Fig ?) Cell borders are lost, but the outline of large c topl sm c areas were clearly dis tinguishable. Abun lant fib illa amyloid substan e (A) is extending close to and even invading the nucleus (arrow) Huge vesicular Golgi zones dense membrane lined bodies of lysosomal morpholo (DL) and a var et of esicular structures are seen



Fig 8



Fig 11
Mesenchymal (Kupffer) cell from amyloidotic mouse liver closely related to invaginations of extracellularly located amyloid (A) Large Golgi area (G) dense mitochondrial and lysosomal structures are further seen

located amyloid fibrils thus causing cellular degeneration with an ultimate total loss of structure

The typical configuration of extracellular amyloid arranged in bundles of fibrils apparently radiating from indentations in the cytoplasm of reticular cells has earlier been noted by *Cohen* (1965) in spleens from amyloidotic rabbits This author too mentioned the often close relationship between such areas and the Golgi zone The stellate arrangement of such bundles emerging from different neighbouring cells (the amyloid star) has been described in experimental amyloidosis in mouse livers by *Heefner & Sorenson* (1962) and by *Gueft & Ghidoni* (1963) The present authors share the opinion expressed by *Cohen* (1965) that the relationship between such bundles of amyloid fibrils and indentations of the cellular surface membrane hardly can be considered suggestive of an act of phagocytosis on part of the cell this view is mainly based on the evident lack of vacuolization and other organized elements characterizing the cytoplasm during a process of phagocytosis

A reasonable explanation of the findings in the present material of a fairly large number of cells showing indisputable intracytoplasmic amyloid may be found in the fact that the experimental model em



Fig 1[illegible]

Sinusoid (S) from amyloidotic mouse liver Sections of surrounding reticular cells and abundant deposits of amyloid material are seen

ployed the C3H mouse of our laboratory very rapidly and with a 100 per cent incidence develops considerable amounts of spleen amyloid in the course of a few weeks of casein treatment Under these conditions at a given moment a relatively larger number of cells will be engaged in the synthesis of amyloid than otherwise being the case in a rather slow experimental model such as the rabbit This will tend to multiply the opportunities of pointing out this particular type of cells in the electron microscope

This feature probably bears some relation to that of the occurrence of the PAS positive cell in experimental mouse amyloidosis (*Teilum* 1956 1964) In a rapidly developing experimental mouse amyloidosis —especially following an acceleration of the disease with *nitrogen* mustard (*Teilum* 1954)-- numerous PAS positive reticular *cells* are found *in juxtaposition* to the *new formed* amyloid substance in a slower model only a few PAS cells are detectable Very probably the amyloid containing cells observed in the present experiment are identical with the PAS cell of Teilum's although technical *difficulties* prevented safe conclusions to be drawn with regard to such an identity

SUMMARY

The results of electron microscopic examinations of spleens and livers from amyloidotic mice indicate apart from the demonstration of numerous cells containing intracellularly located amyloid a certain sequence of degenerative changes in reticular cells apparently related to a shift from an extracellular to an intracellular precipitation of amyloid fibrils in such cells

It appears that one possibility is the secretion of a preamyloid substance which in the extracellular environment is caused to precipitate as bundles of amyloid fibrils arranged in parallel arrays seemingly radiating "out from indentations in the cell border The other possibility is the premature precipitation of amyloid fibrils within these cells before the preamyloid material has managed to reach the cell border This latter mechanism may be looked upon as the consequence of the protracted antigenic stimulation with casein leading to overloading of the cells with preamyloid

Whether the described phenomena of cellular degeneration ultimately leading to total loss of structure of amyloid containing cells is cause or effect of such an alleged mechanism of intracellular amyloid formation remains an open question

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THE SENSITIVITY OF *FLAVOBACTERIUM MENINGOSEPTICUM* TO ANTIBIOTICS AT DIFFERENT TEMPERATURES

An In Vitro Study

By

HELGE OLSEN

Received 2 viii 6

Meningitis caused by *Flavobacterium meningosepticum* is primaril seen in premature infants in which cases treatment has been difficul (*Vandepitte et al* 1958 *King* 1959 *Cabrera & Davis* 1961 *Vandepitte e al* 1965 *Watson et al* 1966)

The microorganism has also been isolated *postoperatively* by bloo cultivation from adults in whom there were no symptoms of meningitis The prognosis in these cases was good (*Olsen et al* 1965)

The sensitivity to a number of antibiotics has been studied *in vitr* for six serologically different strains isolated from patients with *meningitis* The *sensitivity to erythromycin was found to be most in* tense the 50 per cent inhibiting concentration (IC 50) being estimated to range at 3 0–11 µg/ml (*Olsen* 1967)

It has been demonstrated that strains isolated from patients with meningitis are more *temperature resistant* than the above mentioned *strains isolated postoperatively from adults* *These strains do not grow* at temperature above 38 C while the growth of the meningitis strains first ceases at 41° C There is however a slight growth inhibition al ready at 39 C The meningitis strains are *killed after incubation* for two days at 41° C (*Olsen* 1966)

Hyperthermia must thus be expected to have a *favourable effect* of an infection caused by *Flavobacterium meningosepticum* but prema ture infants with meningitis do not often develop fever (*Groover et al* 1961) and therefore do not benefit from the growth inhibiting effect on the microorganism of hyperthermia

Based upon the hypothesis that the slight sensitivity to a ... ties shown by *Flavobacterium meningosepticum* could be incr simultaneous increase *in temperature* the sensitivity to antibiotics has been studied *in vitro* at different temperatu between room temperature (22° C) to 39° C

MATERIAL AND METHODS

Strains Used

The following strains, all isolated from infants with meningitis, were used. The biochemical properties of the strains have been described by *King* (1959) and *Olsen* (1966).

A	King's no 14	ATCC 13253
B	King's no 4[illegible]	ATCC 13254
C	King's no 3375	ATCC 13255
D	King's no [illegible]	
E	King's no 8388	
F	King's no 8707	

1. The sensitivity of the above mentioned six strains to the following antibiotics was studied at the temperatures 35°, 37°, 38° and 39° C: Penicillin G, vancomycin, ampicillin, erythromycin, novobiocin, fucidin, chloramphenicol, pyrrolidino-methyl-tetracycline (Reverin®), streptomycin and sulphadimidine.

A number of two-fold serial dilutions of the antibiotic dissolved in broth was made, each tube containing 0.5 ml. The tubes were inoculated with a platinum loop from a ten-fold diluted broth culture grown overnight at 35° C and two rows were incubated at each of the above mentioned temperatures. The log IC50 was estimated from the number of tubes with visible growth after two days according to the method of Kärber (see for example *Mackie & McCartney* 1962).

The inoculum was estimated by inoculating 0.1 ml of a ten-fold serial dilution produced from the broth culture onto blood agar plates. The inoculum was from 10^4–10 bacteria per tube.

2. 200 µg of erythromycin were added to four tubes containing 10 ml of broth, after which the tubes were inoculated with 0.1 ml of a broth culture grown overnight at 35° C. Then the tubes were incubated at room temperature (22° C), 30°, 37° and 39° C. The number of bacteria was estimated in the four cultures at intervals by the removal of 0.1 ml which was diluted in a ten serial dilution from which amounts of 0.1 ml were inoculated onto blood agar plates.

RESULTS

Tables 1, 2 and 3 show the results according to method 1 for erythromycin, chloramphenicol and penicillin G. A distinct fall in log IC50 was found for erythromycin beginning already in the temperature interval 35°/37°. The fall appeared to be less for chloramphenicol and for penicillin there was an increase rather than a fall. The remaining seven antibiotics were studied using the same number of tests as for penicillin and chloramphenicol.

Table 4 shows the mean values of the fall in $\log_2$ IC 50 for the six strains at all temperature intervals and all ten antibiotics studied. The fall in the titre where P < 0.01 is marked by xx, if P < 0.05 an x is used.

A fall in the log IC50 with increasing temperature was demonstrated for several antibiotics as an expression of an improved effect. The better effect of vancomycin and ampicillin, however, was first seen at 39° C. An increase in log IC50 was found for penicillin G and ampicillin in the intervals 35°/37° and 35°/38°, indicating a poorer effect at higher temperature.

In order to decide whether a similar effect could be demonstrated at temperatures lower than 35° C a test was carried out according to method 2 at 22°, 30°, 37° and 39° C using 20 µg/ml of erythromycin and King's serological type C.

TABLE 1

The Sensitivity Expressed as $\log_2$ IC50 for Six Strains of Flavobacterium meningosepticum (King A–F) to Erythromycin at the Temperatures 3   37   38   and 39 C. Three Tests Were Carried out per Strain The Sensitivity Increased with Temperature

	King A			King B			King C			King D			King E			King F		
35	3 5	4 5	1 5	4 0	3 5	2 5	2 5	3 0	2 5	1 5	2 5	2 0	2 5	1 5	2 5	2 0	2 5	2 0
37	1 5			1 5			1 5			0 5			—0 5			—0 5		
38	0 5	2 0	0 5	1 5	1 5	—1 0	1 5	—1 5	2 5	0 0	—1 5	0 5	–1 5	—1 5	—0 5	—0 5	—0 5	—2 0
39		0 5	1 5		0 5	—3 5		—2 0	0 5		—4 0	—0 5		–2 0	—2 5		—2 5	—3 5

TABLE 2

The Sensitivity to Chloramphenicol Expressed as $\log_2$ IC50 for Six Strains of Flavobacterium meningosepticum *(King A–F) at Temperatures 35 37 38 and 39 C Two Tests per Strain The Sensitivity Increased with Temperature*

	King A		King B		King C		King D		King E		King F	
35	5 5	6 5	6 0	5 5	6 5	6 5	5 5	5 5	5 5	6 0	3 5	3 5
37		6 5		5 5		6 5		5 5		5 5		2 5
38	4 5	5 5	5 5	5 0	5 5	6 5	4 5	5 5	4 5	4 5	1 5	1 5
39	3 5	4 5	5 0	4 5	5 5	5 5	3 5	5 0	4 5	3 5	1 5	1 5

TABLE 3

The Sensitivity to Penicillin Expressed as $\log_2$ IC50 for Six Strains of Flavobacterium meningosepticum *(King A–F) at Temperatures 35 37 38 and 39 C The Sensitivity Was Unchanged or Fell with Increasing Temperature*

	King A		King B		King C		King D		King E		King F	
35	6 0	4 5	5 5	4 5	5 5	4 0	6 0	6 0	5 5	4 5	7 0	5 5
37		4 5		5 5		4 5		6 0		5 5		6 5
38	6 5	5 5	7 0	5 5	5 5	4 5	7 0	6 5	6 0	5 5	7 5	6 5
39	6 5	5 5	6 5	6 5	5 5	4 5	7 5	5 5	5 5	5 0	6 0	4 5

TABLE 4

The Average Fall of $\log_2$ IC50 for Six Strains of Flavobacterium meningosepticum *(King A–F) Studied at Six Temperature Intervals with Ten Antibiotics Where the Average Fall Is Significant at a 1 per Cent Level Is Used at a 5 per Cent Level (The t Test Was Used)*

	Temperature interval					
	35 /37	35 /38	35 /39	37 /38	37 /39	38 /39
Penicillin	—0 58	—0 75	—0.38	—0 25	0 17	0 38
Ampicillin	—0 67	—0 83	—0 54	—0 25	—0 08	0.33
Vancomycin	—0 17	0 42	0 83	0 25	0 83	0 42
Erythromycin	2 00	2 58	4 25	0 42		1 58
Novobiocin	0 58	1 08	2 38	0 75	2 17	1 29
Fucidin	0 17	1 21	2 38	1 00	2 08	1 17
Tetracycline	0 83	1 58	2 54	0 83	1 50	0 96
Chloramphenicol	0 25	0 96	1 50	0 58	1 2	0 54
Streptomycin	0 58	1 08	2 67	0 67	2 33	1 58
Sulphadimidine	1 17	9	3 42	0 83	2 08	1 13

The results are shown in Fig 1 where the time in hours is the abscissa and the ordinate is $\log_{10}$ of the number of bacteria found The number of bacteria fell more quickly at 39 C than at 37 C while the number at 22 and 30 C remained almost constant indicating that 20 µg/ml of erythromycin had a bacteriostatic effect at these temperatures

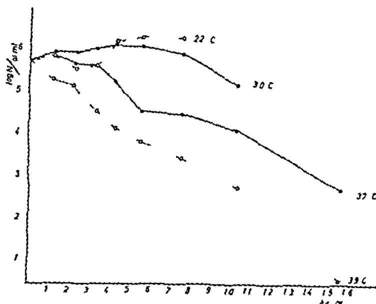


Fig 1
The logarithm of the number of bacteria in broth cultures with 20μg/ml of erythromycin as a function of time at the temperatures 22 30 37 and 39 C. (*Flavobacterium meningosepticum* King's serological type C)

DISCUSSION

With regard to the effect on *Flavobacterium meningosepticum* at different temperatures the ten antibiotics studied can be divided into two groups. Improved effect with increasing temperature was found for erythromycin novobiocin fucidin tetracycline chloramphenicol streptomycin and sulphadimidine. Poorer effect was found for ampicillin and penicillin G. In the temperature interval 38–39 C however any difference was not found in the case of penicillin G and in the case of ampicillin the effect was improved which applies also to vancomycin the effect of which otherwise was independent of the temperature.

An inhibiting effect on the protein synthesis (*Hahn 1966*) has been demonstrated for several of the antibiotics where an improved effect with higher temperature was seen in this study. Another common feature for these antibiotics is that they excluding streptomycin have a bacteriostatic effect.

If the phenomenon demonstrated depended on an increasing number of bacteria being destroyed with increasing temperature independent of the presence of an antibiotic all antibiotics might be expected to have a better effect with increasing temperature. In addition such a destruction should start already at 37 C but in a previous study (*Olsen 1966*) destruction of *Flavobacterium meningosepticum* was not demonstrated at any of the temperatures used here. A lower rate of growth was demonstrated at 22° and 39 C than at 30 35 37 and 38 C but this

does not explain the phenomenon either as the graphs in Fig 1 should then show common features for 22 and 39 C as compared with the graphs at 30 and 37 C However this is not the case

If the increased effect resulted from a purely molecular effect of the antibiotic with increasing temperature according to Arrhenius law approximately the same increase in effect per degree temperature increase would be expected in all the temperature intervals studied here However the effect per degree of temperature increase appears to increase with the temperature Thus any difference in the effect of erythromycin has not been demonstrated in the temperature interval 22 / 30 C but a considerable difference in the interval 35 /39 C This is not consistent with Arrhenius law

Fassin et al (1955) have found similar conditions for *Salmonella typhi* where 3–4 times greater sensitivity was demonstrated for chloramphenicol at 40 than at 37 C

It must be considered whether the phenomenon thus demonstrated can be used therapeutically in cases of severe meningitis in premature infants caused by *Flavobacterium meningosepticum* The microorganism is most sensitive to erythromycin where the IC50 *in vitro* has been found to be 3 0–11 0 μg/ml (*Olsen* 1967) High fever or treatment with hyperthermia must be expected to have a favourable effect on the erythromycin treatment but even under these conditions it is doubtful whether effective concentrations can be obtained in the spinal fluid to which erythromycin has difficulty in passing (*George et al* 1961) treated a child in whom meningitis was caused by *Flavobacterium* meningosepticum by daily administration of 250 mg of erythromycin by mouth but the spinal fluid had only a slight bacteriostatic effect on the microorganism

One author (*Etteldorf* 1955) states that erythromycin has an effect on meningitis caused by pneumococci and streptococci but these bacteria are also more sensitive to erythromycin than *Flavobacterium meningosepticum* even if the improved effect of a higher temperature is taken into consideration

SUMMARY

The sensitivity of *Flavobacterium meningosepticum* to a number of antibiotics was studied at different temperatures Erythromycin novobiocin fucidin tetracycline chloramphenicol streptomycin and sulphadimidine showed an increased effect with increasing temperature while ampicillin and penicillin C showed decreased effect with increasing temperature up to 38 C

It is considered doubtful whether the phenomenon demonstrated can have therapeutic consequences in the treatment of meningitis caused by *Flavobacterium meningosepticum* in premature infants

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AEROMONAS HYDROPHILA S LIQUEFACIENS ISOLATED FROM TONSILLITIS IN MAN

Report of a Case

By

NIELS KOK

Received 21 vi 67

Infection attributable to *Aeromonas* being a rare occurrence in man a report shall be given of a case of tonsillitis in which the therapeutical result as well as findings by bacteriological and serological tests suggested that *Aeromonas* was the pathogenitic agent

CASE HISTORY

Complicated extraction of a molar (-8) was performed on a 30 year old man on November 22 1962 in the surgical department of the Royal Dental College in Copenhagen (reg no 5099 P) Local swelling and pain followed and four months later the affected site had to be excochleated Daily administrations of 1 000 000 i u of di penicillin throughout eight days had no definite effect When swallowing the patient felt pain in the left side of the pharynx and he was more or less fatigued Five months and fifteen days after the extraction of the tooth the patient was referred to the department of general pathology The tonsil in the left side was rather red and swollen the tongue was coated tenderness was manifest behind the mandibular angle there was no lymph node swelling The right side of the pharynx was unaffected *Aeromonas hydrophila* was found to predominate (together with a small number of apathogenic bacteria of the usual oral flora) in cultures of swabs from the left tonsil while growth of this strain was sparse in cultures of swabs from the dorsum of the tongue the strain was not demonstrable in swabs from the tonsil in the right side Negative Paul Bunnell reaction

The isolated *Aeromonas* strain being highly sensitive to chloramphenicol treatment by this compound was instituted but on account of general malaise the patient continued with the drug only for 24 hours

One month later (while the patient was on a sojourn in France) a marked left sided angular lymph node swelling set in response to daily administrations for four days of one gramme of streptomycin prompt and similar symptoms did not appear again one month after treatment *Aeromonas* was not demonstrable either in swabs from the tonsils or from the dorsum of the tongu

RESULTS

Microbiology

Colonies produced by the isolated *Aeromonas* strain after incubation for 24 hours at 37 C on horse blood agar were about 1-2 5 mm in dia

[1] The author wants to express his gratitude to Dr *Bojsen Møller* Statens Serum Institut Dept of diagnostics for determination of species

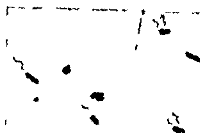


Fig 1
Polar and lateral flagella in the isolated strain of *Aeromonas hydrophila* Leifson's stain

meter they were low dome shaped circular greyish shining with irregular surfaces the rods were gram negative and measured 0.5 × 1 2.5 µm many were slightly curved Staining of the flagella according to *Leifson's method revealed as usual in addition to a polar flagellum* several cells with a lateral flagellum in the initial cultures (Fig 1) this phenomenon has previously been described by *Caselitz & Gunther* (1960) *Leifson & Hugh* (1953) The strain formed acid from aesculin arabinose arginine dextrin fructose galactose glucose (gas at 22 C) glycerol lactose laevulose maltose mannitol salicin sucrose starch and trehalose but did not utilize citrate dulcitol hippurate inositol raffinose rhamnose sorbitol xylose and urea Indol and hydrogen sulphide was formed and nitrate was reduced Voges Proskauer's test as well as oxidase and katalase tests were positive and gelatine was liquefied These results identify the strain as *Aeromonas hydrophila* (*Bergey's* Manual 1957) *type 1* (*Caselitz & Gunther* 1960) or group 1 (*Eddy* 1960 who preferred the name *Aeromonas liquefaciens*)

The strain produced intense beta haemolysis on horse blood agar Seitz filtrates (undiluted and diluted 1 + 24 v/v) of broth cultures aged 24 and 48 hours produced complete haemolysis after 24 hours in suspensions of rabbit erythrocytes (20 ml per liter) but not in suspensions of erythrocytes from man and horse

The sensitivity of the isolated strain to chemotherapeutics was tested by the tablet method (Rosco Ltd) on blood agar It was highly sensitive to chloramphenicol moderately sensitive to streptomycin neomycin bacitracin erythromycin tetracycline and polymyxin B It was fully resistant to sulphathiazol penicillin and fucidic acid

Serology

Eleven months after manifestation of the primary symptoms and three months after the last a single turbid serum sample was obtained Boiled or freeze thawed antigens from the isolated *Aeromonas* strain were used for test tube and agar gel *precipitation* tests (Ouchterlony) and for test tube *agglutination* (24 hours at 37 C) with a wide range of serum dilutions all results were zero

Bacteria from broth cultures aged 24 hours were washed three times in physiological saline and sterilized by heating (70 °C for 60 minutes). An antigen suspension containing 4×10^{4}–5×10^{4} cells per ml was used for *intracutaneous* injections (100 μl). After intervals of 30 minutes and 24 hours, reactions did not deviate from that of a control papule containing physiological saline. Forty-eight hours later similar tests were carried out on the patient and on two healthy subjects; a suspension containing 4×10^{8}–5×10^{8} cells per ml was used. The patient as well as the two subjects had moderate acute reactions, but only the patient had a tuberculin-like reaction after 24 hours. The general condition of the patient remained unaffected, but intense swelling and diffuse reddening of the tested forearms of the two control subjects developed within a few hours; their general condition was influenced and they ran temperatures of 40 °C.

DISCUSSION AND CONCLUSIONS

While infection due to species of the genus *Aeromonas* is not uncommon in poikilothermic animals, reports are few of such infection in man and in warmblooded animals, notwithstanding that these bacteria may produce experimental infection in animals (*Thal & Dinter* 1953, *Thal* 1953, *Caselitz et al.* 1957, *Eddy* 1960, *Hill et al.* 1954, *Bras et al.* 1954, *Kjems* 1955, *Lautrop* 1961, *Caselitz* 1966, *Mattine*, *Silva et al.* 1961).

Although the causal relation cannot be proved beyond doubt in the case discussed here, in which tonsillitis developed after extraction of a tooth, several features seem to substantiate the assumption that *Aeromonas* were responsible for the infection. This genus is seldom present in expectorate under normal conditions, but even so almost pure cultures of *Aeromonas* were obtained by swabs from the surface of the infected tonsil exclusively. In accordance with the pattern of sensitivity, the infection failed to respond to penicillin, whereas the condition improved immediately upon medication with streptomycin. The skin test using a suspension of heat-killed *Aeromonas* provoked a toxic local and general reaction in the two healthy subjects; the reaction provoked in the patient was of a tuberculin-like nature, but he had no toxic symptoms, possibly because of a formation of antihaemolysin (cf. *Thal & Dinter* 1953, *Caselitz et al.* 1958). The absence of demonstrable agglutinin and precipitin may be ascribable to the fact that the serum sample was drawn three months after the disease had subsided.

SUMMARY

A case of tonsillitis in a man has been discussed. From an affected tonsil an almost pure culture of a gram-negative rod was isolated, which according to biochemical tests etc. was classified as *Aeromonas hydro*

TABLE I

Serum Levels in Units per ml Following Oral Administration of One Million Units of Calcium Penicillin V

Patient		Hours after administration											
		Bedridden						Ambulatory					
Sex	No	½	1	2	4	6	8	½	1	2	4	6	8
♂	1	1.8	3.4	1.9	0.38	0.13	0.13	5.8	4.3	0.83	0.67	0.16	0.13
♂	2	0.80	3.2	[illegible]	0.32	0.13	0.13	11	12	4.0	[illegible]	[illegible]	0.13
♂	3	15	9.9	[illegible]	0.32	0.13	0.13	0.16	0.70	1.9	2.1	2.4	1.8
♂	4	0.16	13	8.3	1.5	[illegible]	0.13	≤0.10	0.48	5.9	4.6	2.7	0.90
♂	5	4.6	5.1	[illegible]	5.8	0.86	0.35	[illegible]	3.8	2.7	0.48	0.16	0.13
♂	6	4.0	2.2	4.0	[illegible]	0.13	≤0.10	—	4.2	1.4	0.16	≤0.10	≤0.10
♂	7	8.2	[illegible]	1.8	0.26	0.13	≤0.10	5.8	11	2.7	0.51	0.19	≤0.10
♂	8	7.7	2.2	4.5	0.90	[illegible]	≤0.10	3.7	[illegible]	2.2	0.48	0.16	≤0.10
♂	9	1.6	1.8	1.3	1.0	0.19	≤0.10	[illegible]	3.7	2.6	[illegible]	0.13	≤0.10
♀	10	1.3	[illegible]	[illegible]	1.4	0.26	≤0.10						
♀	11	[illegible]	18	3.8	0.64	0.22	0.10						
		19	22	4.0	1.1	0.29	0.13						
♀	12	12	10	3.2	0.90	[illegible]	0.16						
		13	11	5.0	[illegible]	0.64	0.13						
♀	13	0.54	7.7	2.9	0.98	[illegible]	≤0.10						
		2.4	8.5	3.4	0.74	0.16	≤0.10						

TABLL 2

Serum Levels in Un ' pe ml Foll u ing Oral Administration of One Million Units of Ben athine Penicillin V

Patient		Hours after administration											
		B dridden						Ambulatory					
Sex	No	½	1	2	4	6	8	½	1	2	4	6	8
♂	21	0 45	0 9	0 93	0 14	≤ 0 10	0 13	1 6	1 1	1 0	0 32	0 48	≤ 0 10
♂	22	2 2	1 9	1 4	0 18	≤ 0 10	≤ 0 10	1 3	1 4	0 99	0 77	0 99	0 13
♂	3	1 9	[illegible]	1 7	0 39	0 27	0 13	2 1	1 9	[illegible]	1 0	0 51	≤ 0 10
♂	24	1 9	[illegible]	1 4	0 26	≤ 0 10	≤ 0 10	2 9	1 9	0 67	0 10	0 11	≤ 0 10
♂	25	0 83	1 8	1 2	1 8	1 8	0 48	1 3	1 2	3 2	1 0	0 38	0 14
♂	26	3 7	4 3	1 3	0 38	0 13	≤ 0 10	3 7	3 4	2 7	0 54	0 21	≤ 0 10
♀	27	2 6	1 2	0 69	0 26	0 61	[illegible]	1 2	1 2	0 99	0 35	0 13	≤ 0 10
♀	28	0 77	0 83	0 61	0 45	0 83	0 48	0 38	0 54	0 80	0 45	0 61	0 38
♀	29	1 8	2 9	1 3	0 38	≤ 0 10	≤ 0 10	[illegible]	2 2	1 1	0 30	0 14	≤ 0 10
♀	30	1 0	[illegible]	0 93	0 74	0 19	≤ 0 10	1 3	1 3	1 2	0 42	0 14	≤ 0 10
♀	31	0 91	1 5	2 9	0 99	0 22	≤ 0 10	1 2	[illegible]	1 1	0 96	0 29	0 13

RESULTS

A *Calcium Penicillin V*

Table 1 shows the serum concentrations after administration of one million units Calcium Penicillin V to 13 subjects (9 men and 4 women). One subject participated in only one experiment (as bedridden); 3 were bedridden on both experimental days and the remaining 9 subjects were bedridden on the one and ambulatory on the other experimental day.

1 *Bedridden*

The study comprises thirteen subjects, three of which served twice, resulting in a total of 16 investigations. Five obtained a peak concentration after ½ hour, 9 after 1 hour and 2 after 2 hours. The antibiotic concentrations ranged from 0 16 to 18 units per ml ½ hour after ingestion. At 1 hour they ranged from 1 8 to 22 units per ml and at 2 hours from 1 3 to 8 3 units per ml. The variation increased again at 4 hours and was then 0 26 to 5 8 units per ml, at 6 hours 0 13 to 0 64 units per ml and finally at 8 hours from ≤ 0 1 to 0 35 units per ml.

2 *Ambulatory*

Of the 9 participants in this study, 2 obtained peak values after ½ hour, 5 after 1 hour, 1 after 2 hours and 1 as late as 6 hours. The antibiotic concentrations ranged from ≤ 0 1 to 11 units per ml at ½ hour, from 0 48 to 12 units per ml at 1 hour and from 0 83 to 5 9 units per ml at 2 hours. An increase in variation was noted again at 4 hours and was now from 0 16 to 4 6 units per ml. Serum concentrations ranged from ≤ 0 1 to 2 7 and from ≤ 0 1 to 1 8 units per ml at 6 and 8 hours respectively.

In Table 3 the variation between the serum concentrations is expressed in another way and will be discussed later.

B *Benzathine Penicillin V*

Table 2 shows the serum concentrations after administration of 1 million units of Benzathine Penicillin V to 11 subjects (6 men and 5 women). All the subjects participated bedridden as well as ambulatory.

1 *Bedridden*

Three subjects obtained peak values after ½ hour, 6 after 1 hour and 2 after 2 hours. The antibiotic concentrations ranged from 0 45 to 3 7 units per ml at ½ hour, from 0 59 to 4 3 units per ml at 1 hour, from 0 61 to 2 9 units per ml at 2 hours and from 0 14 to 1 8 units per ml at 4 hours. The ranges were from ≤ 0 10 to 1 8 and ≤ 0 10 to 0 48 units per ml at 6 and 8 hours respectively. In this group the course followed by the serum concentration curves for many of the

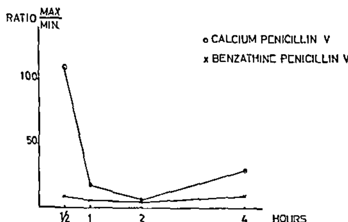


Fig 1
The ratio between maximum and minimum serum penicillin concentrations after oral penicillin V administration

subjects varies so little that it is not possible to estimate the exact time of the peak value

2 *Ambulatory*

Five subjects exhibited peak values after ½ hour and 4 after 1 hour However many subjects had the same levels at both times The largest difference between the serum concentrations occurred at ½ hour and was from 0 38 to 3 7 units per ml At 1 hour the concentrations ranged from 0 54 to 3 4 units per ml and at 2 hours from 0 67 to 3 2 units per ml The difference increased again at 4 hours and was here from 0 10 to 1 0 units per ml Values ranged from 0 11 to 0 99 units per ml and from $\leq$ 0 10 to 0 38 units per ml at 6 and 8 hours respectively

In Table 3 the relationship between the highest and lowest serum concentration found at the different times for the four groups are given The relationship is shown graphically in Fig 1 where the time is the abscissa and the ratio (*i e* max/min at each time) is the ordinate Whereas the ratio for Benzathine Penicillin V is almost a straight line parallel to the abscissa the curve for the Calcium salt reflects the considerable difference between the highest and lowest values at the different times

The individual serum values (in Tables 1 and 2) indicate that no systematic difference exists neither between men and women nor between bedridden and ambulatory subjects following oral administration of penicillin To regard this problem from a different point of view the area under the concentration curves has been determined This is an attempt to express the total amount of penicillin that has been present in the blood during the whole experimental period In other words an attempt has been made to compile the course of the

TABLE

Maximum and Minimum Serum Level in Units per ml and

Penicillin	State of activity	½ Min	½ Max	½ Ratio	1 Min	1 Max	1 Ratio
Calcium Penicillin V	Bedridden	0 16	18	113	1 8	[illegible]	[illegible]
	Ambulatory	≤0 10	11	≥110	0 48	[illegible]	[illegible]
Benzathine Penicillin V	Bedridden	0 45	3 7	8	0 59	4 3	7
	Ambulatory	0 38	3 7	10	0 54	3 4	6

serum concentration curve into a single figure expressing the amount of antibiotic present in the serum of the individual Since however the concentration has been measured at only 6 different times the calculation of the area must be based on an approximate formula

$$A = \frac{(2h_{1/2} + 3h_{1} + 6h_{2} + 8h_{6} + 8h_{8})}{4}$$

where A is the area under the curve and the horizontal axis and h_t is the concentration at time t This formula has been derived by assuming the concentration curve to be made up of a number of straight lines drawn through the observed points and might be considered a good approximation

The results of the calculated areas are recorded in Table 4 In a single case the result is given as > than the observed value This is

TABLE 4

Areas below the Concentration Curves

Patient Sex	Patient No	Calcium Penicillin V Bedridden	Calcium Penicillin V Ambulatory	Patient Sex	Patient No	Benzathine Penicillin V Bedridden	Benzathine Penicillin V Ambulatory
♂	1	4 73		♂	21	1 75	3 11
♂	2	4 57	5 81	♂	22	3 35	4 35
♂	3	12 19	13 89	♂	23	3 79	4 79
♂	4	16 29	>9 98	♂	24	3 65	81
♂	5	18 95	16 13	♂	25	7 26	5 89
♂	6	7 29	7.36	♂	26	5 20	6 34
♂	7	7 28		♀	27	3 44	2 59
♂	8	6 93	10 45	♀	28	3 40	3 93
♂	9	4 15	6 87	♀	29	5 83	3 45
♀	10	7 88	5 77	♀	30	3 05	2 94
♀	11	18 05		♀	31	5.37	3 74
		21 59					
♀	12	13 04					
		16 59					
♀	13	8 09					
		9 12					

r Ratio Following Oral Penicillin V Administration

administration											
2		4			6			8			
Max	Ratio	Min	Max	Ratio	Min	Max	Ratio	Min	Max	Ratio	
63	6	0 ?6	5 8	??	0 13	0 86	6	≤0 10	0 3?	≥4	
59	7	0 16	4 6	29	≤0 10	2 7	≥27	≤0 10	1 8	≥18	
?9	5	0 14	1 8	13	≤0 10	1 8	≥18	≤0 10	0 48	≥5	
3?	5	0 10	1 0	10	0 11	0 99	9	≤0 10	0 38	≥4	

due to the fact that the concentration for pt no 4 is 1 8 units at 8 hours and consequently estimated not to be zero even after 10 hours The results are shown graphically in Fig 2 where A for bedridden subjects is plotted as the abscissa and A for ambulatory subjects as the ordinate It is seen that the two values for the same person lie nearly on the identity line This has been regarded as demonstrating that the area under the concentration curve is characteristic for both the subject and the

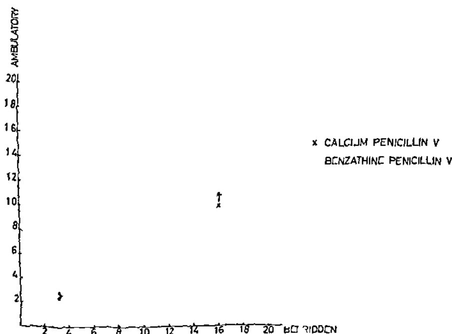


Fig 2

Relationship between estimated total serum penicillin content in bedridden and ambulatory patients after oral administration of Calcium Penicillin V and Benzathine Penicillin V

antibiotic under consideration Furthermore there is no suggestion of systematic difference between bedridden and ambulatory patients

Urinary Excretion

Table 5 shows the urinary recovery over a 24 hour period expressed as per cent of dose given following oral ingestion of 1 million units of Penicillin V The excretion after the Calcium salt ranged for 7 subjects from 19 to 30 per cent with an average recovery of 25 per cent The values for 6 subjects receiving the Benzathine salt ranged from 12 to 20 per cent with an average recovery of 15 per cent

TABLE 5

Percentage of Dose Recovered in Urine in 24 Hours Following Oral Penicillin V Preparations

Calcium Penicillin V Volunteer							Average	Benzathine Penicillin V Volunteer						Average
A	B	C	D	E	F	G		H	I	J	K	L	M	
27	19	22	28	30	26	21	25	20	12	15	15	13	13	15

DISCUSSION

The previously shown difference in serum concentrations between bed ridden and ambulatory subjects following parenteral administration of penicillin has not been shown after oral ingestion

It has been shown however that the solubility of the orally administered penicillin greatly influences the serum concentrations attained Following administration of the sparingly soluble Benzathine salt low but relatively uniform serum levels are obtained throughout the experimental period In contrast the values for the relatively soluble Calcium salt are more variable both between individuals and throughout the experimental period In addition it should be mentioned that the degree of utilization by the organism judged by the urinary excretion is highest for parenterally administered penicillin where ambulatory and bedridden patients excrete 60 to 80 per cent of the given dose respectively After oral administration only 25 per cent of the easily soluble and 15 per cent of the sparingly soluble penicillin are excreted with the urine This difference can be explained partly by a non quantitative absorption from the intestine and partly by the fact that destruction of penicillin in the liver after oral ingestion is more pronounced than after parenteral administration since all the absorbed penicillin is brought immediately to the liver via the portal system The explanation is probably a combination of both factors

The degree of utilization by the organism based on area calculations is greatest after administration of the easily soluble Calcium salt of

Penicillin V. Provided that similar conditions are valid for the absorption of other drugs, relatively insoluble compounds of strongly active pharmacodynamical drugs should be preferred in oral treatment. However, in antibiotic therapy where a high serum concentration if tolerated is required, it would be more appropriate to use an easily soluble compound. It must be realized that larger individual variations will occur both concerning time and size of the peak concentration, but the calculated degree of utilization implies that this is considerably larger after administration of easily soluble salts.

The experiments were performed with penicillin, however it can be presumed that the variations demonstrated here are probably also applicable to other drugs. Hence it follows that the difference in the serum concentrations between good and bad absorbers after ½ hour can be a factor greater than one hundred. This considerable difference may explain either unintentioned or missing effect of several drugs.

SUMMARY

The investigations presented here gave no indication that the activity of the patients should influence the serum concentration after orally administered penicillin. It was shown that the serum concentrations following oral ingestion of an easily soluble salt are higher than those attained with a sparingly soluble salt of penicillin V. However, larger variations occur in individuals receiving the easily soluble salt. The importance of the solubility of drugs is discussed.

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STUDIES ON *YERSINIA ENTEROCOLITICA*

Occurrence in Various Groups of Acute Abdominal Disease

By

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Received 11 x 67

Since the report of *Hassig et al* (1949) several papers have been published on the isolation of *Yersinia enterocolitica* from man (*Carlsson et al* 1964 *Mollaret et al* 1964 *Wauters & Mollaret* 1965 *Winblad et al* 1966 *Graux & Wauters* 1966 *Niléhn* 1967)

Preliminary serological observations (*Winblad et al* 1966) suggested a correlation between *Y enterocolitica* and certain types of acute abdominal disease An investigation was therefore started to find out the incidence of *Y enterocolitica* in patients with acute abdominal pain suggestive of acute appendicitis This investigation which covered a 1 year period was carried out on patients in the Department of Surgery Patients operated upon in the Gynaecological Department children admitted to hospital because of traumatic injury healthy school children and healthy adults served as controls

This paper is concerned with the frequency of positive *Y enterocolitica* culture in the various groups studied the experience gained with the culture method used and characteristics of the *Y enterocolitica* strains isolated Some groups were also examined for agglutinins against *Y enterocolitica* When possible cases where culture gave growth were followed up regarding the development of antibodies and the duration of positive findings in the faeces

MATERIAL AND METHODS

Clinical Material

Patient Groups

1 581 of altogether 601 patients appendectomized in the Surgical Department (1st November–31st October 1966) Culture was performed on the contents of the appendices in all cases and also on the stools in 200 cases In 8 cases lymph nodes extirpated at the operation were received for culture (4 patients with a diagnosis of regional terminal ileitis 3 patients with mesenteric lymphadenitis and one with acute appendicitis and enlarged lymph nodes) Blood samples for serological in

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vestigation taken at the time of operation were obtained from 547 patients 274 of whom were followed up with further samples taken at least 6 days after the operation

2 784 patients admitted to the Surgical Department (1st January 1966–1st December 1966) because of acute abdominal pain not operated upon but in which acute appendicitis was preliminary suspected At least one faecal specimen from each patient was received for culture Blood samples for serological investigation taken in the acute phase of the disease were collected from 124 patients 31 patients could be followed up serologically on samples taken at least 6 days after the day of admission

Control Groups

3 783 women operated upon in the Gynaecological Department (1st December 1965–28th February 1967) because of various gynaecological diseases apparently normal appendices were removed in association with the operations Culture of the contents of the appendices was performed in all cases Single samples for serology were received from 161 patients

4 104 children 3 months–12 years old hospitalized because of traumatic injury (1st January 1966–31st December 1966) Culture from faeces

5 457 healthy school children 7–14 years old (1st November 1966–31st December 1966) Culture from faeces

6 130 healthy adults employed at the hospital Culture from faeces and single serum samples for serological examination

None of the patients or controls had received antibacterial drugs before collection of the samples for culture

The diagnoses in group I were based on observations made at the operation and routine histological examination of the operative specimens Some doubtful cases were re examined histologically by Dr N H *Sternby* Department of Pathology Malmo General Hospital

Patients with both macroscopically and microscopically normal appendices but in whom enlarged mesenteric lymph nodes were observed at the operation were referred to the group of mesenteric lymphadenitis Gross and/or microscopical signs of acute inflammation of the appendix were interpreted as acute appendicitis Distinct inflammation of the most distal part of the ilium was accepted as a sign of regional terminal ileitis In the differentiation between the acute and chronic type of inflammation the criteria set up by *Crohn & Yarnis* (1958) were used

Bacteriological Technique

Media

Lactose saccharose urea agar (LSU agar) was prepared according to *Juhlin & Ericson* (1961) and poured into plastic Petri dishes 9 cm in diameter 28 ml/dish

Human blood agar The medium was poured in two 14 ml layers into Petri dishes as described above Bottom layer 0.5 per cent Bacto Beef extract (Difco Lab Detroit 1 Michigan) 0 [illegible] per cent NaCl 0.2 per cent Na HPO 2H O 1.4 per cent Japan shr d agar (Munthe [illegible] Gothenburg Sweden) H O pH 7.6–7.8 Upper layer the same medium as above with 1 per cent Proteose peptone no 3 (Difco) and 10 per cent human red blood cells added pH 7.6–7.8

Chocolate [illegible] agar was prepared as described by Juhlin (196) and poured into Petri dishes of the same kind as above 28 ml in each

Meat extract broth Bacto Beef extract (Difco) 0 [illegible] per cent NaCl 0.3 per cent Na HPO 2H O 0.2 per cent Bacto peptone (AB Orth [illegible] Kemisk fabrik Copenhagen Denmark) 1 per cent glucose 0.1 per cent H O pH 7.6–7.8 Autoclaved 120 C, 30 minutes Tube 15 × 150 mm 5 ml in each

Cu [illegible] Bacto beef extract (Difco) 0.5 per cent NaCl 0.3 per cent Na HPO 2H O 0.2 per cent glucose 1 per cent Levteinhydro [illegible] (Leo AB Stockholm Sweden) 0.3 per cent H O pH 7.0 7.2 Dispensed into tubes 15 × 150 mm 5 ml in each covered with 1 ml of sterile paraffin autoclaved 10 minutes at 120 C

Rappaport broth Prepared according to *Rappaport et al* (19[illegible] 1959) Tubes 15 × 150 mm 5 ml in each

Culture Technique

The appendix was slit up under sterile conditions and material was collected from the mucosa with a swab. The material was cultured aerobically as streak cultures on human blood agar plates at 37° C, on LSU agar at 22° C and at 37° C, and also in Rappaport broth at 22° C and at 37° C. The latter was incubated for about 48 hours and subcultured to LSU agar 22° C and 37° C respectively. The plates were inspected after one and two days' incubation. Various colonies were isolated for further analysis.

Faecal specimens were cultured in the way described above but human blood agar was used in only a small number of known positive cases. Culture from mesenteric lymph nodes was performed in the following way: the glandular tissue was homogenized under sterile conditions and cultured in the same way as the contents of the appendices with the addition of streak cultures on human blood agar incubated at 22° C on chocolate ascites agar at 37° C and on human blood agar incubated in an anerobic jar at 37° C. The plates were inspected after 1, 2 and 3 days' incubation. The glandular tissue was also cultured in meat extract broth and in cystein hydrochloride broth incubated at 37° C for 2 days.

Bacteriological Analysis

Y. enterocolitica strains were identified and characterized according to methods described previously (Winblad 1967). The technique used for determinations of acid formation from various carbohydrates was modified in only one respect: the concentration of test substances was 1 per cent instead of previously used 0.5 per cent, final pH 7.5–7.8.

In the faecal specimens no search was made for other bacteria except Salmonellae and Shigellae. In culture from the contents of the appendices haemolytic streptococci, staphylococci and suspected pneumococci observed on human blood agar at 37° C were isolated for further investigation. Analysis of the Gram negative flora was made both from LSU agar and from human blood agar to establish as far as possible the diagnosis of present Salmonella or Shigella as well as of Y. enterocolitica if any. In culture from mesenteric lymph nodes any bacteria observed were isolated for further analysis.

Serological Investigation

Patients' sera were investigated for agglutinins against O antigen preparations of Y. enterocolitica prepared according to the technique described by Winblad et al. (1966) with the exception that the bacterial suspension was autoclaved for one hour. Antigen suspensions containing about 10^9 bacteria/ml were used.

RESULTS

I. *Patients Appendectomized in the Surgical Department*

The results of Y. enterocolitica culture in this group in relation to clinical diagnoses, sex and age groups are presented in Tables 1 and 2.

The cultures were positive in 22 out of 381 patients (5.8 per cent). The highest incidence was seen in patients with regional terminal ileitis where the bacterium was isolated in 8 out of 10 patients. In these 8 positive patients who were 9 to 58 years old the disease had been diagnosed by the surgeons as acute terminal ileitis. The other 2 patients were subjected to ileo-caecal resection, one immediately and the other at a second operation about 3 months later. The histological appearance of the operative specimens in both cases fitted in diagnosis of regional granulomatous enteritis (Crohn's (Crohn & Yarnis 1958). In the group "mesenteric lymphaden[illegible] ture gave growth in 9 of 69 cases (about 13 per cent).

cases were seen in patients without any demonstrable lesions and 2 in the group with acute appendicitis No positive culture was obtained in the 72 cases included in the group of miscellaneous diagnoses to which patients with the following diseases were referred acute salpingitis or other gynaecological disease obstruction or perforation of the bowel biliary tract disease inflamed Meckel s diverticulum chronic appendicitis pneumonia and mumps A few cases of appendectomy in association with other operations were also included in this group

TABLE 1

Incidence of Positive Yersinia enterocolitica Culture in 581 Patients Appendectomized in the Surgical Department Malmo General Hospital during One Year (1st November 1965–31st October 1966) No Antibiotics Given at the Time of the Investigation

Diagnosis	Culture	Females		Males		Total	
		neg	pos	neg	pos	neg	pos
Acute appendicitis		145	1	190	1	335	2
Mesenteric lymphadenitis		38	4	22	5	60	9
Regional terminal ileitis		1	3	1	5	2	8
No demonstrable lesions		60	1	30	2	90	3
Miscellaneous		49	0	23	0	72	0
	Total	293	9	266	13	559	22

All diagnosed as acute terminal ileitis

The ages of the patients with positive cultures varied between 4 and 58 years The incidence of positive cultures was roughly equal in both sexes and showed no seasonal variation

The bacterium was isolated from the appendix in all the 22 positive cases except one where it was isolated from faecal samples only Sixteen of the patients showed repeated positive cultures from the stools at investigations carried out after the appendectomy From 4 patients faecal samples were not received

The longest time after the operation that culture gave growth of *Y enterocolitica* was 5 weeks The results of repeated cultures from some patients as well as serological findings are given in Fig 1

Although no detailed epidemiological study of the positive cases was performed family studies showed in 3 cases the occurrence of the bacterium in family members who also had acute abdominal symptoms in one case leading to appendectomy in one case admission to hospital because of suspected appendicitis and in one case because of severe enterocolitis with abdominal pain

Culture from mesenteric lymph nodes from 5 patients in whom *Y enterocolitica* was isolated from the appendix (from 4 with regional terminal ileitis of the acute type and from one with mesenteric lymphadenitis) gave no growth of bacteria But in 3 cases including 2 diagnosed as mesenteric lymphadenitis and one as acute appendicitis

TABLE 2

Incidence of Positive Yersinia enterocolitica Culture in Relation to Clinical Diagnoses and Age Groups in 581 Patients Appendectomized in the Surgical Department Malmö General Hospital during One Year

Age (years)	Total		Acute appendicitis		Mesenteric lymphadenitis		Regional terminal ileitis		No demonstrable lesions		Other diagnoses	
	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos
0 – 4	15	1	6	–	6	1	–	–	1	–	2	–
5 – 9	45	4	15	1	18	2	–	1	6	–	5	–
10 – 14	81	4	52	–	18	2	–	1	12	1	1	–
15 – 19	81	3	44	1	11	1	1	1	16	–	12	–
20 – 24	90	5	52	–	4	3	–	2	23	–	12	–
25 – 29	[illegible]	2	42	–	2	–	1	1	4	1	2	–
30 – 34	28	–	21		1	–			1	–	5	–
35 – 39	30	–	18	–	–	–	–	–	6	–	6	–
40 – 44	26	1	19	–	–	–	–	1	[illegible]	–	2	–
45 – 49	30		16	–	–		–	–	6	–	8	–
50 – 54	[illegible]	1	14		–		–	–	4	1	4	–
55 – 59	19	1	9	–	–	–	–	1	4	–	6	–
60 – 64	10	–	7	–	–		–	–	–	–	3	–
65 – 69	12	–	10	–	–	–	–	–	1	–	1	–
70 – 74	7	–	6	–	–	–	–	–	1	–	–	–
75 – 79	5	–	3	–	–	–	–	–	–	–	2	–
80 –	1	–	–		–		–	–	–	–	1	–
Number	[illegible]	[illegible]	335	[illegible]	60	9	2	8	90	3	72	0
Per cent		3.8		0.6		13.0				3.2		0

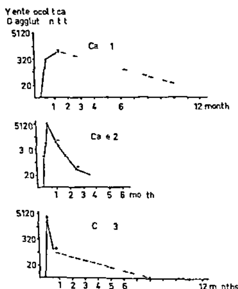


Fig 1

Agglutinin curves and results of culture in three patients from whom Y enterocolitica was isolated Follow up 3–12 months

Case 1 ♀ 8 years old Admitted to hospital because of nausea and pain in right lower quadrant Temp 39 6 C W B C 15 000 Operation revealed a normal appendix oedema and reddening of distal 5 cm of ileum and enlarged mesenteric lymph nodes Histology normal appendix Uneventful recovery

Case 2 ♀ 19 years old One day s history of pain in right lo er quadrant Temp 38 ? C W B C 10 200 Operation revealed a normal appendix Later course uneventful apart from erythema nodosum 1? days after the operation

Case 3 ♂ 14 years old One day s history of pain in right lower quadrant accompanied by nausea Temp 38 6 C W B C 17 000 Chills with temp 40 C next day The following day temperature fell to 36 7 C Not operated upon No recurrence of symptoms

+ Y *enterocolitica* culture positive
— Y *enterocolitica* culture positive

where culture for Y *enterocolitica* was negative culture from lymph nodes gave growth of β haemo ytic strep ococci *Proteus rettgeri* and α haemolytic streptococci r spectively

Though the intestinal flor of the appendices was not analysed in detail β haemolytic streptococci were isolat d from 11 pa i nts with acute appendicitis or mesenteric lymphad nitis *Salmon lla typhimurium* from one patient with acute appendicitis pneumococci from 4 patients 2 with mesenteri lymphadenitis one with t ite appendicitis and or without any demonstrable lesions *Staphylococcus aureus* occurred in 2 ultures both from normal appe dices removed in association with oth operations

Convalescent u i samples taken at least 6 days after the operation w i obtain d from 234 patients The incidence of agglutinins again t Y *enteroc li ica* O antigen preparation among these patients

are presented in Tables 3 and 4. Among the 22 cases with positive *Y. enterocolitica* culture (Table 3), 20 being followed up serologically, showed maximal titres between 1:80–1:5120. One patient, examined only once, showed a titre of 1:40 on the fourth day after the operation. Another patient, in whom no agglutinins were detected at the time of the operation, showed a titre of 1:10 in a second sample collected as late as 3 months afterwards. Fifteen of the patients were followed up for 3–13 months. All of these showed gradually decreasing agglutinin titres. Two patients showed no detectable agglutinins after 4 and 10 months respectively. As a rule, low titres of 1:10–1:20 persisted 4–12 months after the onset of the disease (Fig. 1). One patient, however, had a titre of 1:80 even 13 months after the operation.

Among 214 patients with negative *Y. enterocolitica* cultures (Table 4), only one developed a maximal titre of 1:640, 2 showed titres of 1:160–320, 7 had titres between 1:40–80 and 201 had no detectable agglutinins.

TABLE 3

Incidence of Y. enterocolitica O agglutinins in Various Categories of Appendectomized Patients. Y. enterocolitica Culture Positive

Diagnosis	Total	Maximal titre (inverted values)					
		0	10–20	40–80	160–320	640–1280	2560–5120
Acute appendicitis	2	–	1	1	–		
No demonstrable lesions	3	–	–	–	1	1	1
Mesenteric lymphadenitis	9		–	1§	–	4	4
Regional terminal ileitis	8	–	–	–		5	3
	22	–	1	2	1	10	8

Titre 3 months after the operation. Investigation in the acute phase did not show any detectable agglutinins.

§ Titre 4 days after the operation. Investigation at the time of the operation showed no detectable agglutinins.

TABLE 4

Incidence of Y. enterocolitica O agglutinins in Various Categories of Appendectomized Patients. Y. enterocolitica Culture Negative. Two or More Samples with at least 6 Days Interval Received for Serological Investigation

Diagnosis	Total	Maximal titre (inverted value)				
		0	10–20	40–80	160–320	640
Acute appendicitis	157	147	3	6	1	–
No demonstrable lesions	30	27		1	1	1
Mesenteric lymphadenitis	11	11			–	
Regional terminal ileitis	2	2			–	–
Other diagnoses	14	14	–		–	–
	214	201	3	7	2	1

Single samples for serology were taken at the time of the operation from 312 patients with negative Y enterocolitica cultures One patient with acute appendicitis had a titre of 1 40 Low titres of 1 10–20 were seen in 10 patients with acute appendicitis in 3 patients without any demonstrable lesions and in 4 patients with miscellaneous diagnoses

TABLE 5

Incidence of Positive Yersinia enterocolitica *Culture from Faeces from 284 Patients Admitted to Hospital because of Acute Abdominal Pain not Operated upon No Antibiotics Given at the Time of the Investigation*

Category	Females		Males		Total	
	neg	pos	neg	pos	neg	pos
Symptoms simulating acute appendicitis	157	9	79	7	236	16
Other acute abdominal diseases	26	–	6	–	32	–
Total	183	9	85	7	268	16

TABLE 6

Incidence of Positive Yersinia enterocolitica *Culture in Relation to Clinical Diagnosis and Age Groups in 284 Patients not Operated upon Admitted to Hospital because of Acute Abdominal Pain*

Age (years)	Total		Symptoms simulating acute appendicitis		Other acute abdominal diseases	
	neg	pos	neg	pos	neg	pos
0 – 4	43	1	40	1	3	–
5 – 9	64	3	63	3	1	–
10 – 14	33	2	31	2	2	–
15 – 19	15	3	15	3	–	–
20 – 24	25	5	24	5	1	–
25 – 29	13	–	12	–	1	–
30 – 34	8	–	8	–	–	–
35 – 39	12	–	9	–	3	–
40 – 44	7	–	6	–	1	–
45 – 49	8	1	6	1	2	–
50 – 54	10	–	9	–	1	–
55 – 59	4	1	4	1	–	–
60 – 64	8	–	3	–	5	–
6 – 69	5	–	2	–	3	–
70 – 74	1	–	–	–	1	–
75 – 79	8	–	3	–	5	–
80 –	4	–	1	–	3	–
Number	68	16	236	16	2	–

2 *Unoperated Pat nts with Acute Abdominal Pain Suggestive of Acute Appendicitis*

Fa al samples from altogether 284 patients with symptoms suggesting acute appendicit were examined for Y enterocolitica The results of culture are given in relation to clinical diagnoses sex and age in

Tables 5 and 6 In 32 of the 284 cases other diagnoses were later established: disease of the genito-urinary tract in 15, peptic ulcer in 6, neoplasma of the gastro-intestinal tract in 7, disease of the biliary tract in 3 cases and pneumonia in one case. *Y. enterocolitica* culture was negative in all these patients.

Of the remaining 252 patients with appendicitis-like symptoms 16 were found to have *Y. enterocolitica* in their stools. From 5 of the positive patients *Y. enterocolitica* was isolated repeatedly from faecal specimens obtained during the first week after the onset of the symptoms. At further follow-up stools cultures from these patients turned negative.

The incidence of cases with positive cultures was largely the same during the whole period of the investigation.

In 2 of the 284 patients in this group culture gave growth of Salmonella.

TABLE 7

Incidence of Yersinia enterocolitica *Agglutinins in Patients with Acute Abdominal Pain not Operated upon.* Y. enterocolitica *Isolated from the Stools*

Number of patients with positive culture	Maximal titre (inverted value) after onset of symptoms: 0	10–20	40–80	160–320	640–1280	2560–5120
16*	2§	0	2	2	4	3

* No serological investigation in three cases.
§ Investigated in the acute phase; no serological follow-up during the first 2–3 months from the onset of symptoms.

The incidence of *Y. enterocolitica* agglutinins (maximal titres) in patients with positive *Y. enterocolitica* cultures is shown in Table 7. Three of the 16 positive patients were not investigated serologically. From 2 others only a single serum sample was obtained from each. Both had been taken in the acute phase of the disease and neither showed agglutinins against the O antigen preparation of the bacterium. The agglutinin titre curve and the results of culture in a case followed up for 8 months are shown in Fig. 1.

Among the 236 patients with negative culture 111 were investigated serologically at the time of admission; 99 showed no agglutinins, 6 had titres between 1:10-20, 4 between 1:40-80, one 1:160 and one 1:2560. On serological follow-up of 8 of the cases with detectable agglutinins 3 showed persistently low titres, 4 decreasing titres and one a slightly rising curve with a maximal titre of 1:80.

3. *Patients Operated upon in the Gynaecological Department. Normal Appendices Removed in Association with the Operations*

The results of culture from the contents of the appendices of 283 women in this group are given in *Table 8*. *Y. enterocolitica* was iso-

lated from one patient who also showed a rising agglutinin curve against the bacterium In this case the diagnosis was a left sided ovarian cyst operation had been undertaken at a time when the patient was complaining of abdominal pain after hysterosalpingography The appendix was found to be macroscopically and microscopically normal Single samples for serology were obtained from 161 patients within this group Except for the above mentioned patient only one showed a high agglutinin titre of 1 5120 which gradually decreased to 1 640 about 2 months later Culture in this case was however repeatedly negative Low titres of 1 10–1 40 were seen in 10 patients

TABLE 8

Results of Y enterocolitica Culture in Various Control Groups No Antibiotics Given at the Time of the Investigation

Cathegory	Number	Y enterocolitica culture pos	neg
Women operated upon because of gynaecological diseases culture from normal appendices	283	1	282
Children hospitalized because of traumatic injury	104	–	104
Healthy adults	130	–	130
Healthy school children	457	–	457

Culture from the stools

4 *104 Children Admitted to Hospital because of Traumatic Injury*

Y enterocolitica was not isolated in any case (Table 8) No samples for serology were obtained in this group

5 *457 Healthy School Children*

The investigation which lasted 2 months was carried out on children from 2 d f rent schools Culture was never positive (Table 8) The investigation did not include serological studies

6 *130 Healthy Ad*

F al cultures e ative (Table 7) Single serum samples for serological investig ti n showed titres of 1 40 in 2 cases of 1 20 in one case and of 1 10 in 3 cases Repeated investigation of these subjects did not show any ri e of the agglutinin titre Neither was there any hist of recent abdominal pain or other gastrointestinal symptom

Bacteriology

Y. *enterocolitica* was isolated on 71 occasions from 39 patients. The incidence of isolation from the different media used during the study are presented in Table 9. It is seen from the table that the best results were obtained by incubation at 22° C whether in case of primary plating on LSU agar or in case of enrichment in Rappaport broth. In 24 cases Y. *enterocolitica* was not isolated from media incubated at 37° C, while in all 71 cases at least one of the media incubated at 22° C yielded growth of the bacterium. In 5 cases the bacterium was isolated only from primary streak culture on LSU agar 22° C and in 6 cases only from Rappaport broth 22° C. In 16 cases however the bacterium was lost in Rappaport broth 22° C. In all these cases subculture of the broth yielded heavy growth of Gram negative intestinal bacteria such as *Pseudomonas aeroginosa* or coliform bacteria. Y. *enterocolitica* could be isolated from only about one third of the samples cultured on human blood agar. In the remaining cases the bacterium could not be recognized in the mixed intestinal flora despite frequently abundant growth on the selective primary solid medium.

Twelve of the Y. *enterocolitica* strains isolated during this study have been described in a previous paper (Mehn 1967). The properties of the remaining strains did not differ appreciably from those described. They gave all good growth at both 22° C and at 37° C. On human blood

TABLE 9

Primary Isolation of Yersinia enterocolitica from Faeces or from Appendices in 71 Positive Specimens Received from 39 Patients. Results of Culture on LSU agar at 22° C and at 37° C with and without Previous Enrichment in Rappaport broth (48 Hours) at 22° C and at 37° C respectively. 35 Specimens also Cultured at 37° C on Human Blood Agar as a Non selective Medium

Medium	Incubation temperature	Culture result Positive	Negative	Total
LSU agar	22° C	61§	10	71
Rappaport broth subcultured to LSU agar	22° C	55†	16	71
LSU agar + Rappaport broth subcultured to LSU agar	22° C	71	0	71
LSU agar	37° C	47	24	71
Rappaport broth subcultured to LSU agar	37° C	11	60	71
LSU agar + Rappaport broth subcultured to LSU agar	37° C	50	21	71
Human blood agar	37° C	11	24	35

Primary streak culture

§ Y. *enterocolitica* isolated only on LSU agar 22° C five times

† Y. *enterocolitica* isolated only from Rappaport broth 22° C six times

agar the S form of the bacteria usually formed larger colonies at 37° C than at 22° C but a certain tendency to rough dissociation at the higher incubation temperature was noted. No haemolysis on human blood agar was seen at 22° C; at 37° C a more or less pronounced zone of clearing of the medium was noted around the colonies after 6–10 days' incubation. Results of biochemical tests were largely the same as those described previously (*Nilehn* 1967). With the use of 1 per cent test substance, however, acid formation from l-arabinose was noted on the 1st–3rd day and from d-galactose on the 1st–5th day and somewhat faster at 37° C. As in the previous study, acid formation varied with the incubation temperature also in the case of glycerol and maltose but less so for the latter when 1 per cent test substance was used instead of 0.5 per cent, previously used. Thus acid formation was observed on the 1st–3rd day at 22° C and on the 3rd–10th day at 37° C. The Voges-Proskauer reaction was positive at 22° C and negative at 37° C. The β-galactosidase reaction (ONPG) was positive for all strains and aesculin hydrolysis was negative. No indole producing strains or strains deviating in some other respects where isolated.

The susceptibility to various antimicrobial agents was largely the same as that of strains investigated previously (*Nilehn* 1967).

DISCUSSION

It is clear from the results that the incidence of positive *Y. enterocolitica* culture from the intestinal tract was higher in patients with certain types of acute abdominal distress than in the control groups. The bacterium was thus isolated from 38 out of 865 patients but from only one out of the 911 controls.

In patients with symptoms leading to appendectomy the incidence of positive cultures was 3.8 per cent. The further division of this group into separate entities based on the observations by several surgeons was sometimes uncertain. The difficulty to find objective criteria for a diagnosis of mesenteric lymphadenitis is well known (*Strombeck* 1932, *Masshoff* 1952, *Kiaer* 1960, *Bottiger* 1962). The distinction between normal appendices and those showing only microscopical signs of acute inflammation was not always certain either.

Positive cultures were obtained above all in the group of terminal ileitis followed by the group of mesenteric lymphadenitis. The high incidence with 8 positive patients out of 10 in the group of terminal ileitis was remarkable. Clinically as well as histologically the two negative patients, who were both subjected to ileo-caecal resection, presented a picture of regional granulomatous enteritis (Crohn's disease) (*Crohn & Yarnis* 1958). But the 8 positive patients were not subjected to ileo-caecal resection and in all of them the findings were interpreted by the surgeons as acute terminal ileitis. Differentiation between acute and chronic or subchronic inflammatory conditions of the terminal

ileum may however be difficult at the operation These patients will of course be followed up Several bacteriological and virological studies concerning the aetiology of acute and chronic inflammatory conditions of the lower ileum have been published (*Crohn & Yarnis 1958 Berning et al 1964*) As far as we know apart from intestinal tuberculosis no particular infection has been found to be distinctly related to terminal ileitis either of the acute type or of the chronic granulomatous type (Crohn s disease) Bacteriological as well as serological findings made in this study may speak in favour of the aetiological significance of *Y enterocolitica* in the 8 patients with acute terminal ileitis investigated here

In the group of mesenteric lymphadenitis which was composed almost exclusively of children and young adults samples from 9 out of 69 patients gave growth of *Y enterocolitica* Also high agglutinin titres were seen in most of these cases In acute mesenteric lymphadenitis several infectious agents have been found to play an aetiological role The occurrence of *Yersinia pseudotuberculosis* (Syn *Pasteurella pseudotuberculosis*) in this condition is well documented (*Albrecht* 1910 *Piechaud* 1952 *Knapp & Masshoff* 1954 *Girard* 1959 *Kauer* 1960 *Mollaret* 1960 *Bolliger* 1962 and others) Other bacteria have also been described as aetiological agents (*Calhala et al* 1961 *Huckstep* 1962 and others) as well as adenovirus (*Kjellen et al* 1957 *Hannoun & Mollaret* 1961 *Bell & Steyn* 1962 *Gardner et al* 1962 *Ross et al* 1962 *Potter* 1964) It is reasonable to assume the possibility of various organisms being responsible for inflammation of the lymphatic nodes in this region In the present study the finding of β haemolytic streptococci *Proteus rettgeri*, and α haemolytic streptococci in pure culture from enlarged nodes supports this assumption

Culture was positive in only a few cases in the groups no demonstrable lesions and acute appendicitis The distribution of positive cases thus deviated somewhat from that of the preliminary serological study by *Winblad et al* (1966) But in the latter the groups of patients within similar clinical categories investigated were rather small and a certain selection of the clinical material cannot be excluded

Y enterocolitica culture from the stools was positive in 5 6 per cent of 284 patients admitted to hospital because of symptoms suggestive of acute appendicitis which however subsided without operation Among 32 patients in this group with various final diagnoses such as acute cholecystitis urinary tract disease acute cholecystitis peptic ulcer etc none gave growth of *Y enterocolitica* In the remaining 252 cases no firm diagnoses were possible Patients with positive cultures did not significantly differ clinically from other patients in this group A positive *Y enterocolitica* culture was seen in only one of the controls In this case the bacterium was cultured from an apparently normal appendix removed in association with the extirpation of a left sided ovarian cyst A rising agglutinin titre against *Y enterocolitica* might

indicate a co-existing infection with this microorganism. In another patient likewise operated upon in the Gynaecological Department a high agglutinin titre was found despite of negative culture from the appendix and from the stools. Any explanation of this titre was not found. The findings in a serological investigation of a rather small group of healthy adults did not differ from those made in previous studies of larger groups of healthy subjects (*Winblad et al.* 1966).

The interpretation of existing usually low agglutinin titres in controls is uncertain. They might indicate a previous infection or exposure to the antigen. The degree of cross reacting agglutinins is still obscure. A search for agglutinins in cases that gave no growth of *Y. enterocolitica* did not suggest any appreciable discordance between the results of culture and the occurrence of agglutinins. It is reasonable to suppose that culture may fail in some cases. A false negative culture may be explained in many ways, for instance it may be due to too small a number of bacteria in an otherwise abundant flora, an unsuitable selective medium, or the culture may have been taken too long a time after the acute onset of the symptoms. It was thus noted that cultures from the stools in many positive cases very soon turned negative, also in cases where antibiotics had not been given. Culture at 22° C seemed to be superior to culture at 37° C both on the solid medium used and in Rappaport broth. Enrichment of the material cultured proved to be valuable in some cases, while in others the intestinal flora became predominant over *Y. enterocolitica*.

It seems reasonable to assume a gastrointestinal portal of entry of this infectious organism. Investigations of domestic animals in the surroundings of the diseased persons have so far been negative. Apart from 3 cases in which the bacterium was found in different members of the same families, any connection between patients with positive cultures was not observed. A seasonal accumulation of positive cases was not seen either.

Tested in various biochemical or physiological reactions the *Y. enterocolitica* strains did not differ largely from one other, nor from human strains previously described (*Mollaret* 1967), except for the small variations due to methodological modifications.

No strains of the closely related *Bacterium enterocoliticum* were isolated during this study. Several American reports of the isolation of this microorganism from human sources have been published (*McIver & Pike* 1934, *Schleifstein & Coleman* 1939, 1943, *Gilbert* 1940, *Schleifstein & Clark* 1947). Of special interest is the report by *Schleifstein & Coleman* (1939) of the isolation of this bacterium from a patient with a terminal ileitis. However, according to our present knowledge this bacterium has not been examined systematically regarding its pathogenicity for human beings. Nor has the taxonomic position of this bacterium to our knowl[illegible] been finally settled.

The findings in this [illegible] argue for *Y. enterocolitica* infection as [illegible]

probable cause of acute abdominal disease above all of acute terminal ileitis and also sometimes of mesenteric lymphadenitis or similar conditions with symptoms of acute appendicitis

SUMMARY

581 patients who had undergone appendectomy and 284 patients not operated upon but admitted to hospital because of symptoms simulating acute appendicitis were examined for *Y enterocolitica* in the intestinal tract *Y enterocolitica* was cultured from 22 patients (3 8 per cent) in the first group and from 16 patients (5 6 per cent) in the second group

The bacterium was isolated in one out of 974 controls including 283 women operated upon because of gynaecological diseases 104 children hospitalized because of traumatic injury and 587 healthy children and adults

Positive cultures were noted above all in patients with a clinical diagnosis of terminal ileitis (8 out of 10 patients) the positive cases were all diagnosed as acute terminal ileitis Culture from 9 out of 69 patients with mesenteric lymphadenitis 3 out of 93 with no demonstrable lesions at operation and 2 out of 337 with microscopical signs of acute inflammation of the appendix also gave growth of the bacterium

All patients with positive *Y enterocolitica* cultures followed up serologically developed agglutinins against O antigen preparation of the bacterium usually in high titres

The study covered a period of somewhat more than 1 year No seasonal variation of the bacterial findings was noted Though the portal of entry of the infection probably is the gastrointestinal tract the source of the infection is still obscure

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BILE ACID TRANSFORMATIONS BY MICROBIAL STRAINS BELONGING TO GENERA FOUND IN INTESTINAL CONTENTS

By

TORE MIDTVEDT and ARNE NORMAN

Received 20 xi 67

The main part of the intestinal flora in man and rat consists of micro organisms belonging within the families *Lactobacillaceae* and *Bacteroidaceae* Other microorganisms also occur but in smaller numbers such as *Escherichia coli* coliforms and clostridia (9 21 22)

The normal intestinal flora contains microorganisms capable of transforming bile acids into a variety of metabolites The main reactions are splitting of conjugates elimination of the hydroxyl group at C-7 oxidation of the hydroxyl groups to keto groups at C 3 C 7 and C 12 and reduction of keto groups to both α and β hydroxyl groups (2 5 6) Some of these reactions have been observed in cultures of strains normally present in the intestinal tract Strains of clostridia (15) enterococci (15) *Bacteroides* (4) and one strain of *Aerobacter aerogenes* (17) have been shown to be capable of splitting conjugated bile acids Several strains of *E coli* (16 18) oxidize the hydroxyl group at C-7 to a keto group One strain of *E freundii* (11) contains a dehydrogenase which catalyses both the dehydrogenation of the 3α hydroxyl group and the reduction of the keto group yielding both α and β hydroxyl groups A strain of *Alcaligenes faecalis* (12) isolated from human faeces converts cholic acid into different ketocholanoic acids amongst which 3 7 12 triketo 5β cholanoic acid was isolated Removal of the 7α hydroxyl group from bile acids in anaerobic cultures of lactobacilli isolated from rat and human intestinal contents has been demonstrated (8 14)

The aim of the present investigation was to study the transforma

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Bile Acids and Steroids No 177

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The following systematic names are given to compounds referred to in this report by trivial names cholic acid, 3α 7α 12α trihydroxy 5β-cholanoic acid chenodeoxycholic acid 3α 7α dihydroxy 5β-cholanoic acid deoxycholic acid 3α 12α dihydroxy 5β-cholanoic acid lithocholic acid 3α monohydroxy 5β-cholanoic acid

TABLE 1

The Metabolism of Free and Conjugated Bile Acids by Microorganisms Belonging within Genera often Found in the Intestinal Contents of Man and Rat

Name of microorganism a	Source b	Aerobic (ae) or anaerobic (an) incubations	Splitting of		Transformation of		
			glycocholic acid	taurocholic acid	Cholic acid	Chenodeoxycholic acid	Lithocholic acid
Lactobacillaceae							
Streptococcus faecalis ATCC 8043	ATCC	an	+	+	—	—	—
Streptococcus faecalis M 19	SBI	an	+	+	—	-	—
Streptococcus group D Heazman Williams	SBI	an	+	+	-	—	—
Streptococcus group D I 97	SBI	an	+	+	-	—	—
Streptococcus Manhattan Albany	SBI	an	+	+	—	—	—
Lactobacillus acidophilus 101	HLD	an	—	—	—	—	—
Lactobacillus delbrueckii ATCC 9595	ATCC	an	—	-	-	—	—
Lactobacillus casei ATCC 7469	ATCC	an	—	—	—	—	—
Lactobacillus arabinosus ATCC 8014	ATCC	an	+	+	-	—	—
Lactobacillus brevis CCM 1817	CCM	an	+	—	—	—	—
Eubacterium nitosu Cr 4	IPP	an	+	+	-	—	—
Eubacterium quintum 1361 B	IPI	an	+	+	+	+	—
Eubacterium cadaveris 1098 C	HI	an	+	+	+	+	+
Eubacterium cadaveris 1109	HI	an	—	—	—	—	—
Eubacterium tortuosum 1734 B	HP	an	—	—	-	—	—
Eubacterium aerofaciens 2663	HI	an	—	—	—	—	—
Eubacterium ventriosum 2405	HP	an	+	+	+	+	—
Eubacterium minutum 2760 B	HI	an	+	+	+	+	—
Eubacterium n ... utu n 2895 C	IPI	an	+	+	+	+	—
Eubacterium parvum 1 1 III	HI	an	—	—	+	+	+
Eubacterium lentum 1899 B	HI	an	—	—	+	+	+
Catenabacterium helminthoides 4 3 A	HI	an	+	+	—	-	—
Catenabacterium catenaforme ... 1 88 C	HP	an	—	—	-	—	—
Ramibacterium ramosum 2514	HI	an	+	+	-	-	—
Ramibacterium pleuriticum	HI	an	—	-	—	—	—
Ramibacterium alactolyticum 2?04	HI	an	+	+	—	—	—
Ramibacterium ... *ntium* 1855 A	HI	a	+	+	—	—	—

[illegible]

Ia tota llh t fll s 911 B	III	a	+	+	—	—	—
Ia tol ac ll s apl n l it s 1535 BS	III	an	+	+	—	—	—
I ropionibacteriac ae							
Butyribacterium rettgeri 3331†	IPP	an	+	+	—	—	—
(ynebacteriaceae							
Corynebacterium diphtheroides 2464	IPP	an	—	—	—	—	—
Bacillaceae							
Bacillus cereus 14/11 63	SBI	ae	—	—	+	+	+
Bacillus cereus 14/11 C3	SBL	an	—	—	+	+	+
B icillus subtilis ATCC 6633	ATCC	ae	—	—	—	—	—
Bacillus subtilis ATCC 6633	ATCC	an	—	—	—	—	—
Clostridium butyricum 9–3–60	SBL	an	+	+	—	—	—
Clostridium difficile ATCC 9689	ATCC	an	—	+	+	+	—
Clostridium perfringens CN 1491	SBL	an	+	+	+	+	+
Clostridium perfringens type B	RVC	an	+	+	+	+	+
Cl stridium tetani W W	WW	an	—	—	—	—	—
Clostridium histolyticum W W	WW	an	—	—	—	—	—
Clostridium histolyticum CN 919	SBI	an	—	—	—	—	—
Clostridium C 62	DCR	an	+	+	—	—	—
Inflabilis r tus PC	IPP	an	—	—	—	—	—
Micrococcaceae							
Staphylococcus aureus Wood	SBL	ae	—	—	—	—	—
Staphyl coccus aureus Wood	SBL	an	—	—	—	—	—
Staphyl coccus aureus S 09	BLKS	ae	—	—	—	—	—
Staphylococcus aureus S 09	BLKS	an	—	—	—	—	—
Achromobacteraceae							
Alkaligenes faecalis NCTC 415	NCTC	ae	+	+	—	—	—
Enterobacteriaceae							
Escherichia coli S 618	BLKS	ae	—	—	+	+	—
Es herichia coli S 618	BLKS	an	—	—	+	+	—
Escherichia coli 3901	WW	ae	—	—	+	+	—
Escherichia c li 3901	WW	an	—	—	+	+	—
Kl bsiell pn um niae type A 3409/60	WW	ae	—	—	—		
Klebsiella pneumoniae type A 3409/60	WW	an	—	—	—	—	—

TABLE 1 (cont.)

Name of microorganism a	Source b	Aerobic (ae) or anaerobic (an) incubations	Splitting of		Transformation of		
			glycocholic acid	taurocholic acid	Cholic acid	Chenodeoxycholic acid	Lithocholic acid
Proteus mirabilis 4198	WB	ac	—	—	—	—	—
Proteus mirabilis 4198	BB	an	+	—		—	—
Bacteroidaceae							
Bacteroides fragilis C48	ODH	an	+	+	+	+	—
Bacteroides fragilis NCTC 9343	NCTC	an	+	+	+	+	—
Bacteroides necr[illegible] NCTC 7[illegible]	NCTC	an	+	+	+	+	—
Pseudomonadaceae							
Pseudomonas aeruginosa NCTC [illegible]244	NCTC	ae	—	—	+	+	—
Actinomycetaceae							
Actinomyces israelii B 65/3409	CSC	an	—	—	—	—	—
Cryptococcaceae							
Candida albicans	SBI	ae	—	—	—	—	—

a The names of the strains are those given by the reference laboratories
b Abbreviations *ATCC* American Type Culture Collection Washington DC USA *BLKS* Bacteriological Laboratory Karolinska Sjukh[illegible] St[illegible] Sweden *CCM* Czechoslovakian Collection of Microorganisms Brno Czechoslovakia *CSC* Centraalbureau voor Schimmelcult[illegible] B[illegible] Netherland[illegible] *DCR* Dept of Germfree Research Stockholm Sweden *IPP* Institut Pasteur Paris France *NCTC* National C[illegible] England *ODH* Ontario Dept of Health Toronto Canada *RLD* Research Laboratories for the Dairic [illegible] *RVC* Royal Veterinary College Stockholm Sweden *SBI* State Bacteriological Laboratory S[illegible]na Sweden *WB* Kaptein W Wilhelmsen og [illegible] Bakteriologiske Institutt Oslo Norway
Received as *Bifidobacterium bifidum*
§ Received as *Bifid[illegible]*
† Received as *Eub[illegible]*
An ana[illegible] [illegible] to convert bilirubin [illegible] (7) Tentatively classified as [illegible]

tions of bile acids by strains belonging within genera normally found in the intestinal tract Special attention was paid to the families *Lacto bacillaceae* and *Bacteroidaceae* since these are the most abundant in testinal microorganisms

MATERIALS AND METHODS

Bacteriological Procedures

Strains The microorganisms used and their sources are listed in Table 1 During the experimental period the microorganisms were subcultured in Todd Hewitt broth (Oxoid) (TH broth) and controlled biochemically using certain criteria given in Bergey s Manual (1) or by *Prevot* (19 20) Microscopical examinations and surface cultivations were made to check the culture for purity before and after all tests for bile acid transformation

Medium All the tests for bile acid transformation were made in TH broth Micro bial transformations of bile acids have previously been found to occur in cultures of this medium incubated with single strains and mixtures of intestinal micro organisms (8)

Sterilization of bile acids Labelled bile acids prepared as sodium salts dissolved in 80 per cent ethanol were added to tubes (internal diameter 13 mm height 100 mm) After evaporation of the solvent the tubes were sterilized by autoclaving at 120 C for 20 minutes No destruction of bile acids was observed as a result of this treatment The tubes were carefully shaken after adding 5 ml of sterile broth to give a homogeneous bile salt concentration

Inoculation Inoculation was usually done by introducing 0 01 ml of TH broth culture of the various strains into the tubes containing TH broth and the bile acid to be studied In a few cases the growth of the test microorganisms was retarded in the media containing conjugated bile acids This inhibition was overcome by using inocula of 0 5 ml of TH broth culture

Incubation All incubations were carried out at 37 C for 7 days Anaerobic con ditions were obtained using the pyrogallol method after heating and cooling of the medium (8) Aerobic conditions in the tubes were obtained by continuous shaking All test tubes were inspected daily for growth during the incubation period

Chemical Procedures

Sources of and references to the methods employed for synthesizing 24-C^{14} labelled and unlabelled free and conjugated bile acids have been given in two pre vious publications (8 14)

Isolation of microbial transformation products of cholic chenodeoxycholic and lithocholic acids All tests for the transformation of free bile acids were performed in TH broth cultures with an original bile acid concentration of 10μM After incuba tion the labelled metabolites were extracted from the broth with ethyl acetate after acidification to pH 1 with hydrochloric acid The ethyl acetate extracts were eva porated dissolved in acetone and aliquots taken for thin layer chromatographic (TLC) analysis as described earlier (14)

Determination of splitting of conjugated bile acids The broth cultures were in cubated for 7 days either with 1 mM of taurodeoxycholic acid or with 1 mM of glycodeoxycholic acid The free bile acids were then extracted with ethyl acetate from the mixture as described above and aliquots were taken for TLC analysis (14) Splitting of less than 1 per cent of the conjugates was detected using this technique

RESULTS

Splitting of conjugated bile acids and transformation of cholic cheno deoxycholic and lithocholic acids by various different microorganisms are summarized in Table 1 The metabolites were separated with TLC in different phase systems and the labelled metabolites were identified only by comparison of chromatographic properties with reference com

TABLE 2

Metabolites Formed by Microorganisms Capable of Transforming Either Cholic Chenodeoxycholic or Lithocholic Acid

Name of microorganism		The TLC mobilities of metabolites formed from lithocholic acid (L) chenodeoxycholic acid (CH) and cholic acid (C)
Lactobacillaceae		
Eubacterium quintum 1961 F	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Eubacterium cadaveris 1098 G	L	(3β OH) (3 keto)
	CH	(3β OH 7α OH) (3 keto 7α OH)
	C	monoketo
Eubacterium ventriosum [illegible]405	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Eubacterium minutum 2760 B	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Eubacterium minutum 2895 C	L	0
	CH	(3α OH 7 keto)
	C	monoketo diketo
Eubacterium parvum 171 III	L	(3β OH) (3 keto)
	CH	(3β OH 7α OH) (3 keto 7α OH) (3α OH 7 keto) (3 7 diketo)
	C	monoketo diketo
Eubacterium lentum 1899 B	L	(3β OH) (3 keto)
	CH	(3 keto 7α OH) (3α OH 7 keto) (3 7 diketo)
	C	monoketo diketo triketo
Bacillaceae		
Bacillus cereus 14 11 f3†	L	(3β OH) (3 keto)
	CH	(3 keto 7α OH) (3α OH 7 keto) unknown metabolite
	C	monoketo
Clostridium difficile ATCC 9689	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Clostridium perfringens CN 1491	L	(3β OH) (3 keto)
	CH	(3β OH 7α OH) (3 keto 7α OH)
	C	monoketo
Clostridium perfringens type B	L	(3β OH) (3 keto)
	CH	(3β OH 7α OH) (3 keto 7α-OH)
	C	monoketo
Enterobacteriaceae		
Escherichia coli S 618†	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Escherichia coli 3201†	L	0
	CH	(3α OH 7 keto)
	C	monoketo

TABLE 2 (cont

Name of microorganism	The TLC mobilities of metabolites formed from lithocholic acid (L) chenodeoxy cholic acid (CH) and cholic acid (C)§	
Bacteroidaceae		
Bacteroides fragilis	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Bacteroides fragilis NCTC 9343	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Bacteroides necrophorus NCTC 7155	L	0
	CH	(3α OH 7 keto)
	C	monoketo
Pseudomonadaceae		
Pseudomonas aeruginosa NCTC A7244	L	0
	CH	(3α OH 7 keto)
	C	monoketo

The abbreviations used are (3 keto) 3 keto 5β cholanoic acid (3β OH) 3β hydroxy 5β cholanoic acid (3α OH 7 keto) 3α hydroxy 7 keto 5β-cholanoic acid (3 7 diketo) 3 7 diketo 5β cholanoic acid (3 keto 7α OH) 7α hydroxy 3 keto 5β cholanoic acid (3β OH 7α OH) 3β 7α dihydroxy 5β cholanoic acid

§ Differentiation of metabolites into three groups was performed dihydromonoketo (monoketo) monohydroxydiketo (diketo) and triketo (triketo) derivatives of cholanoic acid

† Same metabolites were formed both in aerobic and anaerobic cultures

pounds (Table 2) In the case of metabolites formed from cholic acid only dihydroxymonoketo monohydroxydiketo and triketo derivatives of cholic acid were differentiated (Table 2)

Splitting of conjugated bile acids Among the 55 strains of micro organisms studied 30 were found to split bile acid conjugates This reaction was the most common of the bile acid transformations investigated All except three of the strains splitt both taurine and glycine conjugates present in the culture media in a bile salt concentration as high as 1 mM The splitting of only glycine conjugates was demon strated in cultures of *Lactobacillus brevis* and *Proteus mirabilis* and only taurine conjugates were split in cultures of *Clostridium difficile*

Oxidation of the hydroxyl group at C 3 Six strains belonging within the families *Lactobacillaceae* and *Bacillaceae* oxidized lithocholic acid to 3 keto 5β cholanoic acid These strains were also able to reduce the 3 keto 5β cholanoic acid to 3β hydroxy 5β cholanoic acid

Removal of the 7α hydroxyl group None of the strains investigated was able to remove the 7α hydroxyl group from either cholic or chenode oxycholic acid

Oxidation of the hydroxyl group at C 7 Fourteen strains belonging in five families oxidized the hydroxyl group at C 7 None of the strains was able to reduce 3α hydroxy 7 keto 5β cholanoic acid to 3α 7β di hydroxy 5β cholanoic acid

Oxydation of the hydroxyl group at C 12 Investigation of metabolites formed from cholic and deoxycholic acids by *Eubacterium parvum* and *E lentum* showed that these microorganisms were able to form metabolites with a keto group at C 12

DISCUSSION

The main microbial transformations of bile acids occurring in human and rat intestinal tract have been demonstrated in cultures of laboratory strains belonging in genera known to be present in the intestine It is of special interest that all these main microbial transformations of bile acids can be produced by strains belonging within the tribe *Lactobacilleae* This investigation has shown that several strains of this tribe were able to split conjugates to oxidize the hydroxyl groups at C 3 C 7 and C 12 and to reduce the 3 keto group to a 3β hydroxyl group but that none of the strains tested was able to 7α dehydroxylate bile acids However we have demonstrated previously that strains of lactobacilli isolated from rat and human intestinal contents are able to 7α dehydroxylate bile acids (8)

Among the strains investigated 17 were able to oxidize the hydroxyl groups at either C 3 C 7 and C 12 In cultures of 14 out of these 17 strains only monoketo derivatives of chenodeoxycholic acid were isolated which shows that the microorganisms are able to oxidize the hydroxyl groups at either C 3 or C 7 None of the 14 strains was able to form metabolites with a keto group at C 12 All the strains capable of oxidizing the hydroxyl group at C-3 were also able to form 3β hydroxy derivatives in THI broth cultures None of the strains oxidizing the hydroxyl group at C 7 was able to form 7β hydroxy derivatives Bile acids with a 7β hydroxyl group are present in human and rat bile These are formed by liver enzymes from 7 keto derivatives and therefore do not need to be formed by microbial enzymes (2)

Most of the genera studied in this investigation contain strains capable of transforming bile acids but the small number of strains studied does not allow conclusions to be drawn regarding any systematic relationship between the classification of the bacteria studied and the pattern of their bile acid transformation

In recent years great interest has been focused on the microbial transformations of bile acids in certain diseases of the digestive system (3) In acute pancreatitis the presence of microbial splitting of conjugates has been demonstrated to occur in the biliary tract (10) The steatorrhea observed in blind loop syndrome may be caused by altered bile acid metabolism due to bacterial overgrowth of the small intestine (13) Splitting of conjugates interferes with the optimal intraluminar micelle formation It is therefore of interest that the present investigation showed that splitting of conjugates is a very common property of intestinal microorganisms

SUMMARY

Fifty five strains belonging within genera often found in the intestinal tract of man and rat were tested for their ability to split conjugated bile acids and to transform cholic chenodeoxycholic and lithocholic acids It was observed that 30 strains split conjugated bile acids and 17 strains transformed the bile acids in several different ways The transformations consisted of oxidations of the hydroxyl groups at C 3 C 7 or C 12 All strains capable of oxidizing the hydroxyl group at C 3 formed 3β hydroxy derivatives None of the strains tested was able to remove the hydroxyl group at C 7

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BRIEF REPORT

ANTIBODY SYNTHESIS BY TRANSFORMED CELLS IN RABBIT LYMPHOCYTE CULTURES

By *J O Lamvik*

This preliminary paper describes experiments which give *evidence* of antibody synthesis and liberation by transformed cells in lymphocyte cultures containing cells *from* sheep red cell immunized rabbits and stimulated *in vitro* with sheep red cells

Material and Methods

Albino rats were immunized against washed sheep red cells by repeated intra venous injections over 6 to 8 weeks Six to nine weeks after the last immunizing doses 7 lymphocyte culture series were prepared from defibrinated heart blood after lymphocyte separation according to a method described by *Coulson & Chalmers* (1964) Two culture series from non immunized rabbits were made at the same time Each culture tube contained 2 5 × 10⁶ cells in 2 5 ml of culture medium com posed of 80 per cent Parker s tissue culture medium (TC199) and 20 per cent pooled normal rabbit serum Most tubes were stimulated with 0 5 ml of 1 per cent washed sheep red cells in TC199 while 2 5 ml of Phytohaemagglutinin (PHA(P) Difco) in 0 5 ml of TC199 or 0 5 ml of TC199 without stimulator was added to some tubes The culture tubes were incubated at 37 C for different culture periods up to 14 days with change of medium on the 4th and the 8th day At the time of harvesting the cells were washed and tested for the ability to form pericellular lytic plaques in sheep red cell monolayers in the presence of complement in small incubation chambers prepared according to *Cunningham* (1965) The culture supernatants were tested for antibodies and the cell suspensions examined for degree of blastoid transformation

Results

No blastoid cells and no plaque forming cells were present in the cell suspensions used for the culture After incubation for 4–6 days blastoid transformation was observed in all antigen stimulated cultures containing cells from immunized rabbits while no definite blastoid change was noticed in the control cultures apart from the PHA stimulated ones which gave blastoid transformation in cultures from all rabbits

After incubation for 6–8 days plaque forming cells were regularly found in all antigen stimulated cultures containing cells from immunized rabbits while none were found in the control culture variants Lytic plaques were only found around enlarged cells

Plaque forming cells were also found but in reduced numbers after 11 and 14 days of culture In these cultures some cells with small excentric nuclei and abundant basophilic cytoplasm were found in the stained preparations

No agglutinins against sheep red cells were found in the culture supernatants before the 6th day of culture Agglutinins were regularly found after 8 days and showed usually a gradual rise in titre in the second week of culture with maximal titre of 16–64 Lytic antibodies against sheep red cells were present in low titres in the culture medium used for all cultures No definite increase in the lytic titre with prolongation of the culture time was observed

Discussion

The results presented indicate that blastoid cells developing in antigen stimulated cultures may acquire the ability to form lytic antibodies against the antigen In the

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same types of cultures agglutinins against the stimulating antigen were liberated into the culture medium but usually it occurred at a stage later than that at which the first cells with lytic ability appeared. It is of interest to note that concomitant with the appearance of agglutinins some cells with an appearance like that of plasmacells were found in the cultures. Such cells were not demonstrated in lytic plaques although cells smaller than blastoid cells were predominating as plaque forming cells in the late cultures.

Conclusion

Cells with ability to form lytic plaques in sheep red cell monolayers and agglutinins against sheep red cells were found in antigen stimulated cultures containing blood lymphocytes from sheep red cell immunized rabbits.

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